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THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA

IN THE UNITED STATES DISTRICT COURT
FOR THE NORTHERN DISTRICT OF CALIFORNIA
SAN JOSE DIVISION

GENENTECH, INC., Plaintiff and Counterclaim Defendant v. THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA, Defendant and Counterclaimant	Case No. 5:10-cv-02037-LHK (PSG) **APPENDIX A TO THE UNIVERSITY OF PENNSYLVANIA'S OPENING CLAIM CONSTRUCTION BRIEF** Part 7 of 27

The University of Pennsylvania submits herewith a copy of prosecution records from United States Application No. 08/525,800, which issued as United States Patent No. 6,733,752.

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APPENDIX A

FILE HISTORY - '752 PATENT

TAB 20

PART 2 OF 3

simultaneously may result in more efficient signal transduction pathways by generating increased signals not only in extent, but also in diversity. Intermolecular association and transphosphorylation of receptor molecules result in an activated heterodimeric kinase which may provide qualitative or quantitative differences in phosphotyrosine sites necessary for cellular substrate binding.

A carboxyl-terminal-deleted p185 and EGFR can still associate into an active complex, but the associated kinase complex is insufficient to mediate cell transformation. This reflects that the increased diversity of signals transmitted through the carboxyl termini of both receptors is crucial for synergistic transformation. In addition, it is reported that the erbB3 is unique among the erbB family members in its ability to couple to Ptdlns3-kinase. We proposed that the related members of the erbB family are each capable of oligomerization in homo- or hetero-specific manners, creating higher order oligomeric structures with complex regulatory interactions. Consequently, heteroreceptor kinase complexes provide a complex docking surface for cellular SH2 proteins and might elicit a markedly different cellular response than homoreceptor kinases. This model explains the synergistic interaction of EGFR and $p185^{c\text{-}neu}$ in M1 cells that result in cellular transformation.

C. Dominant Negative Effect

Heterodimers composed of wild-type EGFR and kinase-inactive p185 have impaired synergistic heteroreceptor signaling. Disabled heteromers abolished the transformed phenotype in living cells, supporting a transregulation hypothesis of EGFR and Her2/neu. Heterodimerization with truncated neu proteins turned off the EGFR kinase, suppressed the function of normal EGFR, including reduced EGF-binding affinity, and inhibited EGF dependent mitogenic responses. Unlike the active heterodimer, the mutant heterodimer may not be able to assemble into a kinase complex with a conformation suitable for ligand binding. Computer modeling has suggested that the kinase-inactive complex has an open conformation instead of a closed substrate conformation for the active form.

The notion that the endodomain with an intact kinase is relevant to ectodomain function is also supported by other studies. Kashles and co-workers showed that the cytoplasmic domain-deleted EGFR displayed only low affinity for ligand binding. In addition, Spivak-Kroizman *et al.* showed that transfected cells coexpressing Her2 with a kinase-negative EGFR resulted in a loss of high affinity for EGF binding. Furthermore, Yarden and colleagues demonstrated that a chimeric receptor of the EGFR extracellular domain fused with a constitutively kinase-active $p185^{neu}$ conferred high-affinity ligand binding to the receptor.

The experimental model of p185 and EGFR interaction suggests a causal relationship between heterodimeric kinase activities, and malignant transformation may also have clinical implications. There are some examples of human malignancies which may involve the aberrant expression of both EGFR and p185, including the overexpression of EGFR and Her2 in a number of tumors of epithelial origin, e.g., thyroid tumors, pancreatic tumors, or endometrical carcinomas. All four members of the erbB family are expressed in some breast cancers (such as SkBr3 cells), and overexpression of perhaps all of these receptors may occur. These observations suggest that EGF and other ligands for erbB family proteins may contribute to the development and maintenance of the malignant phenotype of these tumors by mediating receptor interaction, recruitment, and activation of multiple subsets of signaling molecules.

IX. PROSPECTIVE: PHENOTYPIC REVERSION OF Her2/neu-MEDIATED MALIGNANCY

The relationship of either the oncogenic $p185^{neu}$ or the overexpressed Her2 to cellular transformation has been revealed using mAbs directed against p185 to modulate the expression and activity of this protein. These data were consistent with a direct effect of anti-receptor antibodies on $p185^{neu}$ expression leading to a loss of surface p185 expression and targeted degradation of the oncogenic p185neu protein. Taken collectively, these studies suggested that the continual expression of Her2/neu was necessary for the maintenance of the neoplastic phenotype of neu-transformed

cells. Figure 5 summarizes current approaches to subvert the Her2/neu-mediated transforming phenotype.

A. Biological Inhibition of Antibody against Her2/neu

Mouse monoclonal antibodies raised against B104-1-1 cells expressing rat p185neu react with the extracellular domain of rat p185 proteins. These mAbs have been used for cell surface staining and immunorecipitation to analyze p185 proteins. Drebin *et al.* first described that the anti-neu mAb 7.16.4 caused a phenotypic reversion of transformed cells. Treatment of B104-1-1 cells with anti-p185 antibody 7.16.4 dramatically inhibited the anchorage-independent growth of these cells, a characteristic of transformed cells (Fig. 4). This effect was selective for $p185^{neu}$ since treatment with the same mAb did not affect the phenotype of ras-transformed cells. Further experiments indicated that the reversion of transformation was correlated with the downregulation of $p185^{neu}$ from the cell surface and to accelerate its degradation rate. The removal of the transforming receptor from the cell surface *in vitro* was associated with a reduction in the malignant phenotype and a conversion of the cellular phenotype into a more normal one. Downregulation of $p185^{neu}$ and the transformed phenotype was dependent on the presence of the mAb and was reversible upon removal of the antibody. *In vivo* studies then showed that treatment with anti-neu mAbs was able to significantly inhibit the tumorigenic growth of neu-transformed NIH/3T3 cells implanted in nude mice. The use of two or more mAbs reactive with distinct epitopes of p185neu resulted in an additive effect and complete tumor growth inhibition in 60% of the treatment animals. Our laboratory has also developed a transgenic model to study the preventative effects of anti-receptor antibodies on incipient tumor development. In the transgenic model, animals that expressed the oncogenic *neu* cDNA under the control of the MMTV promoter, which develops tumors at about 30–35 weeks of age, were used. Studies

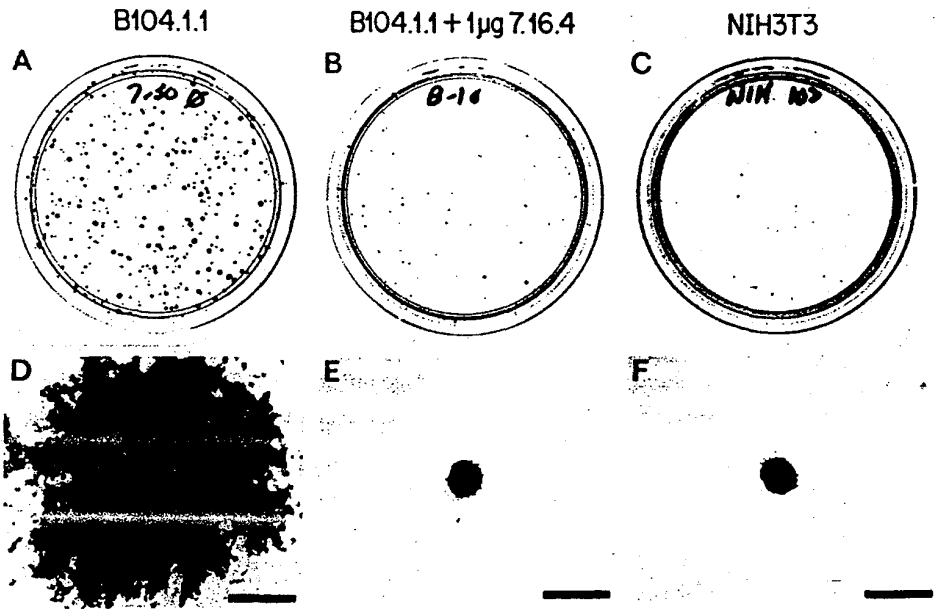

FIGURE 4 Inhibition of soft agar formation by cells transformed by $p185^{neu}$ in the presence of antibody. B104-1-1 cells or NIH/3T3 cells were plated (1×10^5) in 0.18% agarose and fed at weekly intervals with the indicated amount of mAb 7.16.4. Plates were photographed after 2 weeks of growth. (A–C) Photographs of entire culture plates. (D–F) Representative colonies photographed at 40×. [Reproduced from Drebin *et al.* (1985) with permission of the publisher.]

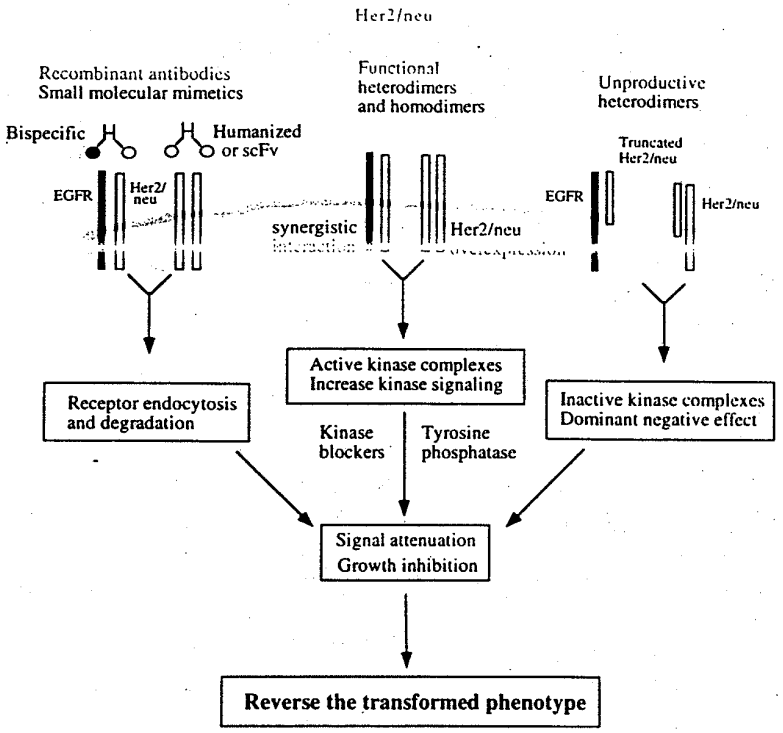

FIGURE 5 Current approaches for the reversion of Her2/neu resultant transformed phenotype (see text for details).

to date indicate that the onset of tumor appearance can be delayed until 50 weeks by the administration of as little as 10 μg weekly of anti-p185 antibody. We have been able to entirely prevent tumor appearance in the transgenics by doubling the amount of anti-p185 antibody administered for over 100 weeks.

The observation that anti-receptor antibodies could revert the malignant phenotype of *neu* and *c-neu*/EGFR-transformed cells *in vitro* and *in vivo* has been widely reproduced and represents a logical approach to cancer therapy. Shepard *et al.* found that one of the anti-Her2 mAbs (4D5) has anti-proliferative or tumor growth-inhibiting activities specific for Her2-overexpressing cells. Other groups have also described similar bioactivities of other anti-Her2 mAbs.

B. Recombinant Antibodies and Small Peptide Molecules

As discussed in the previous section, clinical and experimental evidence supports a role for overexpression of the Her2 protooncogene in the progression of human breast, ovarian, pancreatic colon, and non-small cell lung carcinoma. The overexpressed Her2 present on the surface of human adenocarcinoma cells represents a target for anti-Her2 mAb-mediated immunotherapy. A major limitation in the clinical use of rodent mAb is an anti-idiotypic and anti-globulin response during therapy. One approach to lessen the response is to construct chimeric antibodies by coupling the rodent antigen-binding variable domains to human constant domains. Such chimeric antibody molecules containing a ~30% rodent sequence, however, still elicit a significant anti-globulin response. This potential immunogenicity problem can be further lessened by the humanization of rodent mAb as described first by Winter and colleagues, i.e., grafting the six antigen-binding complementary-determining region loops from their various domains into a human antibody. Using a similar strategy, Carter and colleagues developed a humanized anti-Her2 4D5 mAb and used it clinically for immunotherapy of Her2-overexpressing tumors. In addition, other studies have used humanized bispecific $F(ab')_2$ fragment binding

to both tumor cell antigen (e.g., Her2) and cytotoxic T-cell receptors (e.g., CD3). The lysis of tumor cells appears to be mediated by the release of cytolytic granules from T cells bound to their surface via the bispecific antibody. Furthermore, enzyme-activatable prodrugs in conjugation with humanized antibody–enzyme fusion proteins may enhance the antitumor efficacy of the anti-Her2 antibody and reduce the toxic side effect of conventional chemotheraputics. However, toxicity to the vast number of normal neu-expressed cells in all germ layers may preclude this complex Ab-based therapy. We predict that since neu p185 is found in all secretory epithelial cells, some neural cell toxicity may be unavoidable by an approach using antibody coupled to toxins or drugs. In studies using antibody alone, we found that using combinations of antibodies specific for nonoverlapping domains of p185 were far more potent at reverting phenotypes. Such combined receptor-targeted therapy only affects transferred cells and has no effect on normal p185-expressing cells. Anti-p185 antibodies do not affect p185 expression or function on normal cells. Hence, antibody combination therapy is the most likely to work with the least toxicity.

Other approaches include cloning and expressing recombinant antibodies as single chain Fv (scFv) molecules that exclude an Fc region of the antibody, thus these antibody-derived molecules cannot bind complement or interact with other effector cells. Several scFvs which target tumor cells antigens with similar affinity as their analogous proteolytically produced Fab fragment have been developed. We have been able to clone and express a scFv derived from anti-neu mAb 7.16.4 in bacteria. Bispecific scFvs can also be made to recognize two different receptors on the same cells.

Our previous studies showed that tumors resulting from the synergistic activities of p185 and EGFR (as described earlier) can also be treated *in vivo* with antibodies to either receptor to cause tumor elimination. Consequently, the paradigm that two antibodies can synergistically affect transformation relates to the basic mode of operation of these receptors, namely that they operate as homo- or heterodimeric forms. Our data indicate that it may be more efficient to target two separate domains of these receptor forms than relying on the specificity of a single antibody. This can be achieved by two antibodies reactive with distinct regions, either on the same molecule or on either of the receptor monomers, or by creating a novel bifunctional species of antibody that simultaneously binds both receptor monomers.

Therapeutic compounds designed based on antibodies can be made even smaller than scFv forms. Evidence from biochemical and structural studies of antibody–antigen interactions suggests that an isolated CDR may be sufficient to bind an antigen and retain bioactivity similar to its parental antibody. We have succeeded in using a general synthetic strategy to develop organic CDR structures that lack peptide-bonded amino acids, are poorly immunogenic, are resistant to peptidases, and are nontoxic. These compounds, so-called mimetics, behave in an identical biological manner to the immunoglobulin or CD4 proteins from which they were modeled. Their affinity of interaction with the respective ligands is in the low micromolar range (~4 μM). Recently, we have developed the technology to design small peptides based on the coding sequence of anti-neu mAb 7.16.4. Peptides representing CDR3 of the mAb retained biological activity of the intact molecules specific for growth inhibition and $p185^{neu}$ downregulation in B104-1-1 cells. These findings reflect the potential clinical application of the designed small molecules.

C. Tyrosine Kinase Blockers and Tyrosine Dephosphorylation

Overexpression or a critical mutation results in uncontrolled signaling of the receptor tyrosine kinases. Thus inhibitors that block the activity of tyrosine kinase and signaling pathways they activate may provide a useful base for drug development. The natural inhibitor herbimycin A irreversibly blocks the tyrosine kinases of Src, EGFR, and Her-2. Some synthetic inhibitors, such as tyrphostin, also selectively inhibit tyrosine kinases. The small molecular size of Laverdustin analogs such as AG957 allows them to permeate cells. AG1478 is highly selective for the inhibition of EGFR kinase in the nanomolar range, while AG825 inhibits the Her2 kinase 60 times as efficiently as it inhibits EGFR *in vitro*. Tyrphostins also synergize with Abs to EGFR or Her2 to block the growth of human cells that overexpress Her2 and/or EGFR.

The receptor tyrosine kinase activities result in high levels of autophosphorylation and substrate phosphorylation. It is noteworthy that tyrosine phosphorylation in the cell is a reversible, dynamic process. Protein tyrosine phosphatases (PTPs) are responsible for the removal of phosphate from tyrosine residues (dephosphorylation). An imbalance between these enzymes may impair normal cell growth, leading to cellular transformation. Several studies have reported the inhibitory effects of PTPs on cellular transformation in tissue culture systems. Expression of the human PTP1B gene has been shown to block Her2-mediated cellular transformation. The mechanism of suppression of transformation by PTP1B may be by either a direct effect on Her-2/neu or through an effect on downstream substrates. PTP1B could also partially revert transformation by Src. More comprehensive studies are needed to define the substrates critical for the transformed phenotype and a degree of substrate specificity for PTP *in vivo*.

D. Antagonists for the Action of Her2/neu

We have successfully shown that the preferential heterodimerization of truncated kinase-inactive neu with EGFR suppresses neu and EGFR function and abolishes synergistic transformation. Ullrich and colleagues have demonstrated that glioblastoma growth was effectively inhibited *in vivo* by a retrovirus encoding a dominant-negative FlK-1 mutant, which interfered with the vascular endothelia growth factor receptor. We plan to use folate receptor-mediated gene delivery targeted to breast cancer-bearing transgenic mice expressing oncogenic neu and some neuo/glioblastoma animals expressing Her2neu and/or EGFR at elevated levels. Our recent *in vitro* data showed that expression of a truncated neu (Fig. 3) could also inhibit tumor growth by high efficient codimerization with oncogenic p185neu. In addition, the delivery of truncated neu cDNA into the human glioblastoma cell line U87MD significantly inhibited focus formation and tumor cell growth. Thus, the truncated form of Her2/neu is able to codimerize with either full-length Her2/neu protein or EGFR, inhibit the receptor kinase signaling, and subvert the transfomed phenotype.

See Also the Following Article

PROTEIN TYROSINE KINASES.

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EXHIBIT C

INVITED REVIEW

The p185c-neu/c-erbB-2 growth factor receptor: signal transduction pathways, transformation mechanisms and potential therapies

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A variety of pathways exist to transmit biological signals. One mechanism used for the regulated control of cell growth and differentiation is through the transduction of signals resulting from the binding of soluble polypeptide growth factors to their cognate receptors. The specificity of growth factor action is mediated by the interaction of ligand with cognate receptors which can lead to exquisite control in a tissue- and developmental-specific manner. In addition, individual receptors on the cell surface can form complex assemblies with other receptor/signal transduction molecules that potentially lead to additional levels of signal transmissions. Biological signaling by peptide ligands can be mediated through the enzymatic activation of the receptor resulting in the triggering of a defined biochemical pathway. Ultimately, a mitogenic or differentiation signal is delivered to the nucleus, completing the biological action of the growth factor. The biochemical mechanisms of signal transduction by the p185 neu/c-erbB-2 growth factor receptor and the subsequent physiological responses are the topics of this review.

Study of the p185 growth factor receptor has helped to illustrate the functional role of receptor homo- (and hetero-) dimerization in enzyme activation and, in malignant cells, the detrimental results of structural mutations or aberrant gene expression which may effect this dimerization. The ability of one type of growth factor receptor to affect the activity of another (as illustrated by the p185/epidermal growth factor receptor heterodimeric complex) is likely to be a common regulatory feature of growth factor receptor action.

The nomenclature to be used in this review will refer to the oncogenic mutated form of the rat protein as 'p185^{neu}', the proto-oncogenic rat protein as 'p185$^{c\text{-}neu}$' and the human form as 'p185$^{c\text{-}erbB\text{-}2}$'. The term 'p185' will be used to refer to any type of protein, regardless of the source.

Introduction

The *neu* gene was initially identified as an activated oncogene using DNA transfection analysis. Treatment of pregnant BDIX rats with the carcinogen ethylnitrosourea (ENU) at day 15 of gestation led to the formation of neuro/glioblastomas in the offspring. Purified DNA from these tumors was able to induce malignant growth of NIH 3T3 cells after transfection (Shih *et al.*, 1979; Shih *et al.*, 1981), suggesting that this DNA contained gene(s) with oncogenic potential. In order to identify the protein that was responsible for this transformation, secondary neuroblastoma transfectants were injected into mice and crude antisera from tumor-bearing mice was shown to precipitate a phosphoprotein of 185 kDa present in the transformed neuro/glioblastoma cell lines, but not control cells (Padhy *et al.*, 1982).

These observations suggested that the transforming oncogene directly encoded or somehow induced expression of this 185 kDa protein. Monoclonal antibodies raised against cells transformed with the neuroblastoma oncogene definitively identified an unique 185 kDa protein and specifically reacted with the cell-surface of these transformed cells as well as the original neuroblastoma (Drebin *et al.*, 1984). There was no reactivity with normal NIH 3T3 cells or cells transformed with the *ras* oncogene.

The transforming oncogene was next shown to be similar to existing oncogenes and growth factor receptors in a series of experiments by Schecter and colleagues and was named '*neu*' for the neuro/glioblastoma from which it was derived (Schechter *et al.*, 1984). Southern blot analysis of genomic DNA indicated a high degree of similarity with the *v-erbB* oncogene, and the 185 kDa tumor antigen shared some immunologic determinants with the epidermal growth factor receptor (EGFR) as judged by studies with polyclonal antibodies. Immunoprecipitation with the appropriate antibodies was used to demonstrate that both the p185 tumor antigen and the EGFr co-existed in normal Rat-1 cells. The combination of molecular transfection and biochemical assays unequivocally established that the p185 protein was the product of the *neu* oncogene (Drebin *et al.*, 1984). Thus, this gene provided the first evidence of a tumor antigen which was directly related to a malignant phenotype. The issue of linkage of the expression of tumor antigens with transforming genes was initially approached using *ras*-transformed cells (Drebin *et al.*, 1982) or other oncogenes (Hopkins *et al.*, 1981).

The molecular cloning of the *neu* cDNA was described by Weinberg and colleagues (Bargmann *et al.*, 1986b; Hung *et al.*, 1986). Using related *v-erbB* probes under low stringency hybridization conditions, cosmid clones containing genomic EcoRI fragments were isolated from secondary neuroblastoma cell lines and these cloned fragments were capable of transforming fibroblasts after transfection. The normal allele was also isolated from BDIX rat liver DNA and was not demonstrably different from the activated oncogenic allele by restriction analysis, suggesting only a subtle difference between the two alleles. Moreover, a specific anti-p185 monoclonal antibody (7.16.4) immunoprecipitated a similar 185 kDa protein from cells transfected with either the oncogenic or normal alleles indicating that gross structural alterations of the p185 protein were not observed as a result of oncogenic activation (Stern *et al.*, 1986). Similar DNA hybridization probes were used to demonstrate that the p185-encoding mRNA was present in the *neu*-transformed cell-lines (but not *ras*-

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transformed cells) and facilitated the cDNA cloning from a library constructed from B104-1-1 cells. DNA sequencing indicated that the rat cDNA encoded a 1260 amino acid protein with considerable similarity to the EGFr (as will be discussed below), however, the chromosomal location of the *neu* gene was distinct from the EGFr gene (Schecter *et al.*, 1984).

A comparison of cDNA clones isolated from both normal and tranforming alleles indicated that the difference between the oncogenic and proto-oncogenic form of *neu* is a single point mutation (T -> A) resulting in an amino acid substitution (Val -> Glu) at position 664 within the transmembrane domain of the receptor protein (Bargmann *et al.*, 1986a). This oncogenic substitution was identified in 4 transforming *neu* cDNAs (isolated from separate neuroblastoma cell-lines) and was the only mutation found in the entire cDNA sequence. The critical role of this observed mutation in *neu* on the functional activation of the p185 receptor tyrosine kinase will be addressed below.

The human homologue of *neu*, termed *c-erbB-2* or *HER-2*, has also been identified and characterized (Coussens *et al.*, 1985; King *et al.*, 1985; Semba *et al.*, 1985) using molecular cloning techniques. The human *c-erbB-2* gene was isolated from a human genomic DNA library screened under low stringency condtions with a viral *v-erbB* hybridization probe. The chromosomal locus for *c-erbB-2* was localized to bands q12 -> q22 of chromosome 17. Sequence analysis of the cDNA (Coussens *et al.*, 1985; Yamamoto *et al.*, 1986) confirmed that the *c-erbB-2* gene was the human homologue of *neu* and had significant homology with the EGFr. Although the oncogenic point mutation identified in the rat *neu* has not been found associated with human neoplasias to any degree, the human protein is overexpressed in a variety of adenocarcinomas as a result of *c-erbB-2* gene amplification, and a number of studies have suggested that overexpression of p185 was linked to the neoplastic process. Observation of *c-erbB-2* amplification was first described for a human gastric tumor (Yamamoto *et al.*, 1986; Akiyama *et al.*, 1986). $p185^{c\text{-}erbB\text{-}2}$ overexpression also appears to be associated with non-small cell lung (Kern *et al.*, 1990), colon (Cohen *et al.*, 1989a), ovarian (Slamon *et al.*, 1989), and pancreatic adenocarcinomas (Williams *et al.*, 1991). Overexpression of $p185^{c\text{-}erbB\text{-}2}$ has been found in a significant percentage of breast carcinomas (Slamon *et al.*, 1989; and reviewed in Jardines *et al.*, 1993). Biochemical mechanisms by which $p185^{neu}$ or $p185^{c\text{-}erbB\text{-}2}$ mediate cellular transformation will be discussed below.

The relationship of either the oncogenic p185neu or the overexpressed $p185^{c\text{-}erbB\text{-}2}$ to cellular transformation has also been revealed by using monoclonal antibodies (mAbs) directed against p185 to modulate the expression and activity of this protein. Some of these mAbs have the ability to inhibit the growth of cells transformed by *neu*/*c-erbB-2*, and, therefore, may be potential reagents for the development of immune-based therapies of cancers which overexpress $p185^{c\text{-}erbB\text{-}2}$. Drebin and others first described the effect of an antibody directed against the the rat $p185^{neu}$ protein on $p185^{neu}$ expression in the transformed B104-1-1 cell-line (Drebin *et al.*, 1985).

These experiments demonstrated that the mAb induced a rapid down modulation of surface $p185^{neu}$ which was reversed upon antibody removal. An examination of mAb effects on the transformed phenotype of *neu* oncogene-transformed cells showed an antibody dose-dependent inhibition of anchorage-independent colony formation (indicating a mAb-mediated reversion of the transformed phenotype). Anti-$p185^{neu}$ mAbs also significantly inhibited the growth of p185neu-induced tumors xenoexplanted into athymic mice (Drebin *et al.*, 1986). These data were consistent with a direct effect of anti-receptor antibodies on $p185^{neu}$ expression leading to a loss of surface p185 expression and targeted degradation of the oncogenic $p185^{neu}$ protein.

Other groups have also described similar bioactivities of anti-$p185^{c\text{-}erbB\text{-}2}$ mAbs (Harwerth *et al.*, 1993; Kasprzyk *et al.*, 1992; Maier *et al.*, 1991; Shepard *et al.*, 1991), with some demonstrating anti-proliferative or tumor growth inhibiting activities specific for $p185^{c\text{-}erbB\text{-}2}$-overexpressing cells. Taken collectively, these studies suggested that continual expression of p185 was necessary for the maintenance of the neoplastic phenotype of *neu*-transformed cells, and confirmed the hypothesis that the expression of oncogenic p185 can be functionally linked to cellular transformation.

Primary structure of the p185 growth factor receptor

The amino acid sequence deduced from the rat *neu* and the human *c-erbB-2* cDNAs revealed 1260 and 1255 amino acid proteins, respectively, that are extremely similar to other members of the epidermal growth factor receptor family (Ullrich and Schlessinger, 1990). This family also includes the more recently characterized $p160^{c\text{-}erbB\text{-}3}$ (Kraus *et al.*, 1989) and $p180^{c\text{-}erbB\text{-}4}$ (Plowman *et al.*, 1993) proteins. Proteins in this family have several characteristic features including an extracellular, ligand binding domain which contains 2 cysteine-rich subdomains and several potential sites for glycosylation. Each protein also has a single amphipathic transmembrane region, a short juxtamembrane portion followed by the tyrosine kinase domain and the carboxyl-tail. This kinase domain is contiguous within approximately 300 amino acids and shows the highest degree of amino acid homology between EGF receptor family members. The carboxy-termini of each receptor is more varied in size and sequence, but typically contains the major sites of tyrosine autophosphorylation for each protein.

The structure of the p185 receptor protein fits into this organizational scheme with only a few exceptions. The extracellular portion is homologous (at the amino acid level) with the EGFr (44%) (Bargmann *et al.*, 1986b). The kinase domain is very highly conserved within the family, raising the question as to the foundation of substrate specificity for each receptor kinase. This region exhibits the highest degree of amino acid sequence similarity between each of the members of the EGFr gene family (> 80%). On the basis of protein sequence comparison, Hanks (Hanks *et al.*, 1988) has postulated that subregions within the tyrosine kinase domain confer this type of specificity. Other regions of the cytoplasmic domain (including the juxtamembrane region and carboxy-tail) have been shown to be important for the relatively high transforming activity of p185neu (see below). The carboxyl-terminal residues demonstrate the highest sequence variance between each member of the EGFr family and contains a very high percentage of proline, glycine and serine residues in p185, perhaps allowing this portion of the protein to adopt an unusual structure important for substrate association or enzyme activity. There have been no reports of the 3-dimensional structure of any members of the EGFr family of tyrosine kinases. Detailed structural analysis of the kinase domain will help to delineate any unique protein subdomains important for the signaling specificity of each type of tyrosine kinase.

Enzymatic Regulation of p185

The activity of the p185 tyrosine kinase (and the physiological response to the growth factor) can be controlled at several different levels. This section will describe some of these mechanisms including allosteric enzymatic modulation and regulation of enzyme activity through the control of steady-state levels of p185 expression. The critical role of receptor dimerization and tyrosine phosphorylation on p185 activity will be discussed separately below.

Regulation by serine/threonine phosphorylation

Regulation of EGFr activity can be conferred by phosphorylation of tyrosine, serine, or threonine residues. Protein kinase C activation regulates EGFr (as well as other cellular proteins) via serine/threonine phosphorylation resulting in the down modulation of high affinity binding sites and the concomittant reduction in tyrosine kinase activity (Hunter *et al.*, 1984; Davis and Czech, 1985;). Phorbol esters [such as phorbol-12-myristate-13-acetate (TPA)] activate protein kinase C directly, and can regulate kinase activity and mimic its affect on cellular metabolism. By analogy to the EGFr, p185 can also be regulated by these covalent modifications. TPA has been shown to stimulate phosphorylation of p185$^{c\text{-}erbB\text{-}2}$ in a gastric cancer cell-line MKN-7 (Akiyama *et al.*, 1988). Dobashi *et al.*, (Dobashi *et al.*, 1989) compared the differential effects of TPA treatment on the normal and oncogenic forms of p185 expressed in the same cellular background. Serine and threonine phosphorylation was increased on p185$^{c\text{-}neu}$, but not p185^{neu}. These differential effects on p185 serine threonine phosphorylation correlated with the decrease in tyrosine kinase activity of p185c-neu and the TPA-induced inhibition of cell proliferation. These results indicated that the p185^{neu} receptor species was refractory to serine/threonine kinase regulation. Cao (Cao *et al.*, 1991) also observed an increase phophorylation of p185$^{c\text{-}neu}$ (but not p185^{neu}) by TPA, however a decrease in kinase activity was not observed.

Evidence for other physiological effects of serine/threonine phosphorylation on p185$^{c\text{-}neu}$ has been illustrated by Lee et al. (Lee *et al.*, 1989). In this study, phorbol ester treatment of cells which expressed a chimeric EGFr extracellular domain/p185 intracellular domain protein caused a decrease in the number of high affinity EGF binding sites, mimicking the down modulation effect seen with phorbol ester treatment of EGFr. It has also been demonstrated that prolonged treatment of cells with TPA prevents the induction of immediate early genes that normally follows p185 activation (Lehtola *et al.*, 1991). It is noteworthy that the one defined protein kinase C phosphorylation site in EGFr (Thr 654) appears to be conserved in both p185^{neu} and p185$^{c\text{-}erbB\text{-}2}$ proteins.

Regulation of p185 biosynthesis, internalization, and degradation

The signaling activity of growth factor receptors can also be regulated by the appropriate cell-surface targeting of newly biosynthesized receptors and the rates of internalization and receptor recycling (Brown and Greene, 1991; Sorkin and Waters, 1993). Initial characterization of the *neu* gene product as a cell-surface antigen (Drebin *et al.*, 1984) suggested that this location is important for ligand binding and receptor function. Flanagan and Leder (Flanagan and Leder, 88) explored the importance of cell surface expression of p185 by creating a fusion of the heavy chain of an immunoglobulin (Ig) with the intracellular domain of p185. Cell-surface localization of the p185 kinase domain was then regulated by co-expression with the corresponding Ig light chain. Cellular transformation was dependent on the cell-surface expression of the p185 kinase, presumably through the association of critical substrates partitioned near the plasma membrane. Hudziak and Ullrich (Hudziak and Ullrich, 1991) deleted the transmembrane region of p185 and found that the mutated protein was synthesized to normal levels, but incorrectly processed and retained in the endoplasmic reticulum. Interestingly, this construct still maintained some transformation potential when overexpressed. The mechanism or relevance of this latter observation to normal function was not determined and, in both of these studies, neither the level of p185 kinase activation nor the association of cellular substrates were examined.

Regulation of p185 activity may also be conferred at the level of receptor stability and internalization. It has been shown that the full-length oncogenic p185 receptor has a considerably shorter half life (1.5 hr) as compared to the p185$^{c\text{-}neu}$ (> 7 hr) (Stern *et al.*, 1988) possibly a reflection of the enhanced kinase activity of the oncogenic p185. Internalization of p185 has also been examined by swapping domains with the EGFr to create chimeric receptors (Sorkin *et al.*, 1993). These analyses demonstrated that p185 was less efficient at ligand-induced internalization than wild type EGFr and this retardation was imparted by the carboxyl-terminus of p185.

The stability of p185$^{c\text{-}erbB\text{-}2}$ was also examined in proliferating mammary epithelial cell-lines by Kornilova et al. (Kornilova *et al.*, 1992). Turnover was modulated not only by the growth status of the cells but also by the addition of EGF to the cells. This latter mechanism (which increases the internalization of p185) was presumably mediated by the functional interaction of the EGFr and p185 receptors (see below). Qian and colleagues (Qian *et al.*, 1994a) have utilized mutants of p185 that are kinase inactive to demonstrate dominant negative physiological effects on EGFr function. These studies illustrate that p185 and EGFr both regulate the activity of the other protein at the level of cell-surface internalization and degradation.

The biosynthesis of the p185 protein may also affect the activation of the intrinsic tyrosine kinase and subsequent cellular signaling. A number of investigators have characterized the presence of a soluble polypeptide fragment of p185 that is secreted into the cell culture medium. This phemomena was observed in the sera of animals with p185-mediated progressively growing tumors (Greene et al., unpublished observations) and initially characterized in conditioned media from the breast cancer cell-line SK-BR-3 which overexpresses p185$^{c\text{-}erbB\text{-}2}$ to very high levels (Alper *et al.*, 1990). A 130 kDa fragment was identified as an extracellular domain protein which was immunologically indistinct from p185$^{c\text{-}erbB\text{-}2}$ (Lin and Clinton, 1991). The identity of the 130 kDa form as a fragment of p185 has been confirmed in a number ofexperiments (Mori *et al.*, 1990) (Langton *et al.*, 1991; Leitzel *et al.*, 1992). Pupa et al. (Pupa *et al.*, 1993) inhibited the release of soluble p185 from breast cancer cell-lines by pretreatment with protease inhibitors, suggesting that the specific proteolysis results in the production of this fragment, in aggreement with an independent analysis (Zabrecky *et al.*, 1991).

A truncated p185$^{c\text{-}erbB\text{-}2}$ mRNA has been observed by Scott *et al.* (Scott *et al.*, 1991) in various breast cancer cell-

lines. The truncated transcript apparently arises by alternative RNA processing and leads to the production of secreted and intracellular forms of a 100 kDa polypeptide. These studies of the differential processing of the p185 protein might have implications for p185 regulation. A soluble form of the receptor may potentially compete with endogenous ligand, effectively lowering the physiological response of those cells or it may affect other p185 receptors (Kashles *et al.*, 1991) or the EGFr protein (Qian *et al.*, 1994a) in *trans*. Moreover, the release of a soluble form may also have important implications for the diagnosis of cancer patients or the efficacy of immunotherapies directed against the extracellular domain of p185 (Scott *et al.*, 1993).

Dimerization/enzyme activation

It has been suggested that activation of the growth factor receptor's intrinsic tyrosine kinase follows receptor oligomerization. This mechanism of activation might be prefered to a mechanism of signal transduction through a single receptor molecule, which has been argued to be energetically unfavorable (Ullrich and Schlessinger, 1990). A model for receptor activation by oligomerization has been supported by studies with the EGFr. Receptor oligomerization may be favored by ligand binding which induces a conformational change in the extracellular ligand binding domain. Ligand-induced receptor dimerization of EGFr is believed to provide an allosteric regulatory signal that co-incidentally couples with kinase activation (Canals, 1992). The extracellular domain has been demonstrated to be sufficient for dimer formation of either EGFr or p185 (Lax *et al.*, 1991; Spivak-Kroizman *et al.*, 1992).

Transmembrane domain

The oncogenic potential of the mutated rat p185^{neu} protein appears to be absolutely dependent on the oligomerization mechanism for activation and subsequent signaling. A comparison of normal and oncogenic p185 proteins by non reducing electrophoresis demonstrated that the single amino acid mutation (664E) within the transmembrane domain of p185 causes the protein to dimerize (Weiner *et al.*, 1989b). The oncogenic p185 was shown to be more highly phosphorylated than the protoconcogenic form on tyrosine residues in intact cells (Stern *et al.*, 1988) and more active as a kinase in immune complex kinase assays (Bargmann and Weinberg, 1988) resulting in increased phosphorylation of cellular substrates (Weiner *et al.*, 1989a). To directly test the linkage between the tyrosine kinase activity and cellular transformation, Weiner and colleagues altered the ATP binding site within the kinase domain of oncogenic p185^{neu} by changing lysine 757 to a methionine via site directed mutagenesis. Transfected cell lines expressing this protein were not transformed demonstrating that a functional tyrosine kinase is necessary for the transforming potential of p185^{neu} (Weiner *et al.*, 1989a).

Bargmann (Bargmann *et al.*, 1988) constructed additional point mutations within the transmembrane region to examine their biochemical and cellular effects. Substituted glutamic acid residues at positions 663 or 665 which flank the site of oncogenic alteration did not have any effect on kinase or cellular transforming activity. Several different amino acid changes at position 664 also had no effect, with the exception of Gln 664 which resulted in a transformed phenotype of cells identical to that seen with the Glu 664 substitution. Segatto and co-workers (Segatto *et al.*, 1988) demonstrated that an aspartic acid at position 659 of the human p185$^{c\text{-}erbB\text{-}2}$ (analogous to position 664 in the rat p185) increased the oncogenic potency.

Two alternative hypotheses have been suggested to explain the critical function of amino acid 664 in the oncogenic activation of p185. Brandt-Rauf (Brandt-Rauf *et al.*, 1990) calculated the minimal conformational energy of p185 transmembrane peptides and determined that a peptide containing the transforming alteration (664E) is predominately α-helical, while the nontransforming (664V) peptide contained a sharp bend. Transmembrane α-helices could form dimers readily within the lipid bilayer, thereby promoting dimerization of the entire molecule.

The second hypothesis is based on sterochemical modeling of the transmembrane region and postulates that this region is α-helical in both forms, and that the mutant (664E) residue could form inter-receptor hydrogen bonds resulting in p185 dimerization (Sternberg and Gullick, 1989). The 3-dimensional structure of synthetic p185 transmembrane peptides was determined by NMR and showed both forms to be a-helical (Gullick *et al.*, 1992).

In an important test of the function of the p185 transmebrane region, Lofts and colleagues (Lofts *et al.*, 1993) demonstrated that short transmembrane segments coexpressed at the cell-surface with the oncogenic form of p185 slowed the growth of these cells and inhibited transformation. Inhibition of receptor dimerization was not demonstrated directly, however. Introduction of an additional cysteine residue proximal to the transmembrane domain increased the propensity of either oncogenic or protooncogenic forms of p185 to form dimers, but had no affect on the transforming activity (Cao *et al.*, 1992b). However, artificial dimerization of p185 by inappropriately placed disulfide bonds may not form the proper conformation necessary for p185 activation and transformation.

Another strategy to examine the biological consequences of the oncogenic amino acid substitution in p185 was utilized by Ben-Levy and co-workers (Ben-Levy *et al.*, 1992) in which the ligand binding domain of EGFr was fused to either normal or oncogenic p185 proteins. Cells expressing the chimera which contained the oncogenic neu transmembrane region were transformed and presented only a single high affinity binding population for the ligand (EGF).

Interestingly, the corresponding mutation within the transmembrane region of the EGFr did not effect the enzyme activity or transformation potency of this related tyrosine kinase receptor (Kashles *et al.*, 1988). A mutational analysis by Cao *et al.* (Cao *et al.*, 1992a) demonstrated the importance of neighboring residues in the transmembrane region of p185neu. This data suggested that the local environment proximal to the 664 residue is critical for the oncogenic activation of p185, as previously postulated (Sternberg and Gullick, 1990), and this environment may not exist in the EGFr. Irregardless of the exact mechanism by which the single amino acid affects cellular transformation, it is clear that the mutation causes the p185^{neu} proteins to dimerize more efficiently (Weiner *et al.*, 1989b). Increased oligomerization leads to activation of the p185 tyrosine kinase and enhanced cellular signaling in a manner similar to ligand-induced dimerization and activation of other growth factor receptors. Receptor-receptor oligomerization by the p185 protein is an important mechanism for the receptor tyrosine kinase auto- (and trans-) regulation processes. This mechanism may also be used by other members of the EGFr gene family.

Oligomerization of p185 and malign. ransformation

The relationship between overexpression of the *c-erbB-2* gene and human cancers is persuasive (as described above). An analogous amino acid substitution (V664E) within the p185c-erbB-2 transmembrane region has not been identified, probably since it would require the coordinate mutation of 2 nucleotides to alter the human codon encoding valine to a glutamic acid. However, the role of *c-erbB-2* amplification in human cancer can be explained by a model in which there is an equilibrium between the monomeric and oligomerized forms of the p185 protein. As the quantity of $p185^{c\text{-}erbB\text{-}2}$ protein increases by overexpression, the equilibrium is shifted to the oligomeric state resulting in activation of the tyrosine kinase and inappropriate cellular signaling.

Evidence for this model stems from *in vitro* transformation assays using cell-lines overexpressing the protooncogenic and oncogenic forms of the rat *neu* gene and the human *c-erbB-2* gene. Using focus formation, anchorage-independent growth, and tumor formation in athymic mice as the transformation criteria, studies have shown that *c-erbB-2* will mediate transformation when expressed at high levels in NIH 3T3 cells (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987). Overexpression of the proto-oncogenic form of rat *neu*, using SV40 promoter-driven expression vectors, failed to cause transformation of NIH 3T3 cells (Hung *et al.*, 1986) or NR6 cells which are devoid of endogenous EGFR (Kokai *et al.*, 1989). Apparently, a critical level of overexpression was necessary to acheive a significant growth advantage and transformation of NIH 3T3 cells, since transformation was achieved when LTR-based expression vectors were used (Di Marco *et al.*, 1990). The more powerful LTR promoters allowed 20-fold higher expression levels of $p185^{c\text{-}neu}$ relative to SV40-based systems. A study by Chazin *et al.* (Chazin *et al.*, 1992) confirmed that overexpression of $p185^{c\text{-}erbB\text{-}2}$ by LTR-driven vectors was sufficient to transform cells in the absence of EGFR. However it should be pointed out that the level of expression was extraordinary.

The overexpression of rat *neu* under control of the mouse mammary tumor virus (MMTV) promoter in transgenic mice has been used as a means to analyze the effects of *neu* overexpression in breast epithelia *in vivo*. Overexpression of the oncogenic rat *neu* was sufficient to induce breast adenocarcinomas in a single step (Muller *et al.*, 1988). Although overexpression was detected in a few other tissues, neoplasia did not result, suggesting a role of other mammary-specific factors in adenocarcinoma development. An additional study of transgenic mice overexpressing rat *neu* indicated that breast adenocarcinoma development was a stochastic event requiring additional genetic events for mammary carcinogenesis (Bouchard *et al.*, 1989). Expression of the proto-oncogenic $p185^{c\text{-}neu}$ in transgenic mice resulted in the appearance of focal mammary tumors after a long latency (Guy *et al.*, 1992a). These mammary tumors expressed elevated levels of p185 with an activated tyrosine kinase.

Using the normal and oncogenic form of rat p185 purified from transfected insect cells, LeVea (LeVea *et al.*, 1993) demonstrated that the oncogenic form has a higher propensity to form oligomers as measured by sucrose density gradient centrifugation. In a direct test of the equilibrium model of p185 activation, increasing the concentration of the normal $p185^{c\text{-}neu}$ protein shifted the equilibrium towards the aggregated state (Samanta *et al.*, 1994). Moreover, analysis of enzyme kinetics indicated that the oligomerized pro. s had a significant increase in V_{max} relative to the monomeric protein (LeVea *et al.*, 1993). Non covalently-linked p185 dimers with elevated autophosphorylation activity have been identified in breast cancer cell lines, supporting this hypothesis (Lin *et al.*, 1992).

Taken together, these studies indicated that the inappropriate expression of the normal form of $p185^{c\text{-}erbB\text{-}2}$ can mediate aberrant cellular signaling and oncogenic transformation both *in vitro* and *in vivo*. Moreover, overexpression of $p185^{c\text{-}erbB\text{-}2}$ or the normal form of the rat *neu* ($p185^{c\text{-}neu}$) is functionally linked to the transformation of cells *in vitro* and establishes a potential role for *c-erbB-2* overexpression in human malignancies.

Activation of p185 by other mechanisms

In addition to overexpression and the transmembrane mutations, other structural alterations have activated the oncogenic potential of p185. The transforming efficiency of the proto-oncogenic rat $p185^{c\text{-}neu}$ was increased by a deletion of most of the amino-terminus of the protein in two independent studies (Segatto *et al.*, 1988; Bargmann and Weinberg, 1988). The specific tyrosine kinase activity of these mutant p185 forms was increased, suggesting that the extracellular domain exerts a negative effect on the kinase.

Autophosphorylation of p185

One of the initial consequences of ligand binding to the EGFr is receptor autophosphorylation at discrete sites in the carboxyl-terminus. The proliferative growth signals transmitted by growth factor receptors absolutely require the intrinsic tyrosine kinase activities of these receptors and subsequent autophosphorylation (Ullrich and Schlessinger, 1990). It is unclear why the autophosphorylation levels of activated growth factor receptors exceed the phosphorylation of any other cellular substrates. Receptor autophosphorylation of the EGFr family of receptors does not directly affect the kinase V_{max}, but rather serves to competitively inhibit substrate phosphorylation (Honegger *et al.*, 1988). Guy and colleagues (Guy *et al.*, 1992b) examined the enzyme kinetics of $p185^{neu}$ and $p185^{c\text{-}neu}$ proteins lacking most of their extracellular domains and determined that the increased tyrosine kinase activity of p185neu was partially due to an enhanced utiliztion of Mn^{2+} as a cofactor in phosphorylation. A purified, soluble form of the kinase domain was also demonstrated to use Mn^{2+} more efficiently than Mg^{2+} (Myers *et al.*, 1992). Importantly, in this latter study, autophosphorylation of p185 increased the initial rate of tyrosine kinase and slightly decreased the K_m values for an exogenous substrate.

Autophosphorylated tyrosine residues have been shown to serve as high affinity binding sites for cellular proteins containing src-homology 2 (SH2) domains, thus modulating cellular signal transduction (Reviewed in Koch *et al.*, 1991). The SH2 proteins can be either substrates themselves, or serve as "adaptors" for the assembly of additional proteins on the activated growth factor receptor complex. Considerable specificity of SH2-growth factor receptor interaction is achieved by virtue of the sequence surrounding the phosphorylated tyrosine suggesting a mechanism for exquisite specificity (Birge and Hanafusa, 1993).

Analysis of p185 indicates an extremely high level of receptor autophosphorlyation in the oncogenic, trans-

membrane-mutated form and a high, constitutive level when the proto-oncogenic p185$^{c\text{-}neu}$ or p185$^{c\text{-}erbB\text{-}2}$ forms are overexpressed (Stern et al., 1988; Lonardo *et al.*, 1990). Specific sites of tyrosine autophosphorylation have been identified exclusively within the carboxyl-tail of the p185 protein. Margolis and colleagues (Margolis *et al.*, 1989) localized certain sites of p185 tyrosine phosphorylation to the carboxyl-terminus. Direct analysis by tryptic peptide mapping of *in vitro* labeled human p185$^{c\text{-}erbB\text{-}2}$ protein led to the specific determination of several autophosphorylation sites (Hazan *et al.*, 1990). Tyrosines at positions 1023 and 1248 were unequivocally identified as autophosphorylation sites by protein microsequencing, and additional sites at position 1139 and 1222 were identified by comparison of p185 and EGFr phosphorylation patterns. Collectively, these studies illustrated that the overall autophosphorylation pattern was similar between EGFr and p185. However, one position (Y1086) was uniquely phosphorylated on EGFr, while one site (Y1023) is phosphorylated on p185 but not EGFr. This analysis suggested that certain signaling pathways may be unique or shared between these two receptor tyrosine kinases.

The critical role of autophosphorylation in the cellular transforming activity of p185 has been illustrated by mutational analysis of p185 tyrosine residues by site-directed mutagenesis. Segatto *et al.* (Segatto *et al.*, 1990) altered several tyrosine residues or made larger structural deletions in the proto-oncogenic human p185$^{c\text{-}erbB\text{-}2}$ to examine the function of these residues. Individual amino acid substitutions at positions Y1248, Y1197, Y1139, or Y877 (numbering according to Coussens *et al.*, 1985) failed to abrogate the transforming activity of p185$^{c\text{-}erbB\text{-}2}$ when overexpressed to high levels (using a Maloney MuLV LTR promoter) in NIH 3T3 cells. While individual mutations were reported to have no discernible effect, the formation of focal growth on monolayers was decreased in cells expressing multiple mutations. These effects correlated with an overall reduction in p185 phosphotyrosine content, but were not due to differences in *in vitro* autophosphorylation activity or receptor turnover.

A chimeric molecule containing the EGFr extracellular domain and the intracellular portion of p185$^{c\text{-}erbB\text{-}2}$ was constructed so that the physiological function of these autophosphorylation sites could be examined in a ligand (EGF)-inducible fashion (Segatto *et al.*, 1992). The alteration of multiple tyrosine residues severely impaired the EGF-induced DNA synthesis of transfected cells, indicating that p185-mediated signal transduction was compromised. The *in vitro* kinase activity was unaltered, however, an examination of one putative p185$^{c\text{-}erbB\text{-}2}$ substrate phospholipase C gamma (PLCγ) suggested that autophoshorylation serves to increase the affinity for cellular substrates (see also below).

Using similar techniques, Akiyama *et al.* (Akiyama *et al.*, 1991) examined the function of tyrosine phosphorylation in the human p185$^{c\text{-}erbB\text{-}2}$ protein also containing a transmembrane mutation (V659E) similar to that seen in the oncogenic rat p185^{neu}. A single change of Y1248F reduced the transformed morphology and the foci number of cells expressing this protein. This reduction in transformation was correlated to a decrease in tyrosine kinase activity on an exogenous substrate (a *src* peptide), suggesting a positive role of this tyrosine residue on cellular signaling and transformation.

The analagous residue (Y1253) was also demonstrated to be critical for the transforming activity of the oncogenic rat p185^{neu} protein (Mikami *et al.*, 1992) as assayed by foci formation and tumorigenesis in athymic mice. The magnitude of transformation inhibition correlated with the loss of additional tyrosine residues in a deletion mutant (Tapstop). However, there were no detectable alterations in the tyrosine kinase activity of these mutants towards an exgenous substrate, suggesting that the difference in transformation potencies were correlated to the inablility of substrates to associate efficiently with mutated p185. Collectively, these studies indicated that several critical tyrosine residues are important for conveying p185 mitogenic signals as exemplified by Y1253 in the rat protein (Y1248 in p185$^{c\text{-}erbB\text{-}2}$). The importance of other phosphorylated tyrosine residues has yet to be clarified.

Cellular signaling m\echanisms/substrate association

The activation of kinase substrates leads to the release of second messengers via inositol phosphate and diacylglycerol intermediates. Eventually, signal transduction affects the nuclear transcription of genes which regulate the cell cycle progression. The lack of a cloned ligand for p185 has hindered the discovery of cytosolic substrate proteins for the p185 tyrosine kinase. To reveal biochemical pathways, biological effects, or proteins associated with p185, many investigators have utilized a number of alternative strategies such as ligand induction of an EGFr/p185 chimera or the use of the constituitively activated oncogenic p185^{neu}. One important concern with the studies utilizing the chimeric protein is that the extracellular domain of p185 may be critical to form certain receptor assemblies on the cell surface needed for physiological signaling. These studies with the chimeric proteins may be flawed because of the failure to appreciate the ability of the extracellular domain to drive homo- as well as hetero-dimer formation.

In order to study the function of the p185 tyrosine kinase, Sistonen *et al.* (Sistonen *et al.*, 1989) constructed a chimeric protein consisting of the EGFr extracellular domain and the p185 transmembrane and intracellular domains and expressed the protein in NIH 3T3 cells. The chimeric protein was functionally responsive to ligand treatment as demonstrated by rapid autophosphorylation after EGF addition. The activated p185 kinase was capable of inducing the expression of *c-jun*, glucose transporter, and ornithine decarboxylase (ODC) mRNAs. This study emphasized the ability of p185 to transmit signals necessary for transcriptional activation (fos/jun), nutrient transport and cell-cycle progression. To identify any differences between the genetic responses to the ligand activated p185 or the EGFr, Koskinen *et al.*, (Koskinen *et al.*, 1990) examined the differential expression of 78 serum inducible genes in NIH 3T3 cells. There were very few qualitative or quantitiative differences in the mRNAs induced, suggesting that the signaling pathways of the two growth factor receptors converge at the nucleus.

The similarities between EGFr and p185 nuclear signaling raise the question as to the nature of signal transduction specificity, if any, between these two proteins. Presumably, the tissue-specific expression of either protein on different cells or ligand availibility would comprise two levels of specificity, but are there also inherent differences in the biochemical coupling of the activated tyrosine kinases? The specificity of substrate recognition or availibility of these substrates could also confer additional controls on ligand signaling specificity.

Although EGFr and p185 signaling does appear to converge at the nucleus, the observed patterns of tyrosine

Table 1. Proteins which interact with the p185 tyrosine kinase

Protein	*Physical interaction*	*Tyrosine phosphorylation*	*Enzymatic activity*	*References*
epidermal growth factor receptor	+	+	+	Wada *et al.*, 1990; Goldman *et al.*, 1990; Qian *et al.*, 1992; Spivak-Krioizman *et al.*, 1992
phospholipase C-gamma (PLCγ)	+	+	N.D.	Fazioli *et al.*, 1991; Peles *et al.*, 1991; Segatto *et al.*, 1992; Jallal *et al.*, 1992
GTPase activating protein (GAP)	+	+	N.D.	Fazioli *et al.*, 1991; Jallal *et al.*, 1992
phosphatidylinositol-3'-kinase (PI-3'-kinase)	+	?	+	Scott *et al.*, 1991; Peles *et al.*, 1992[a]
phosphatidylinositol-4'-kinase (PI-4'-kinase)	+	N.D.	+	Scott *et al.*, 1991
Shc	+	+	N.D.	Segatto *et al.*, 1993
c-src tyrosine kinase	+	N.D.	+	Muthuswamy *et al.*, 1994; Luttrell *et al.*, 1994
protein tyrosine phosphatase (PTP 1C)	+	N.D.	+	Vogel *et al.*, 1993
protein tyrosine phosphatase (PTP 1D)	+	N.D.	-	Vogel *et al.*, 1993
unidentified 56 kDa protein	+	+	N.D.	Scott *et al.*, 1991

N.D., not determined; [a] in this study the tyrosine phosphorylation of either subunit of PI-3'-kinase (110 kDa or 85 kDa) was not shown directly.

phosphorylated proteins in cells NIH 3T3 cells transformed by these two tyrosine kinases are qualitatively and quantitatively different (Di Fiore *et al.*, 1990a). p185 also had a significantly higher transforming potencies than EGFr in NIH 3T3 cells, but, in a hematopoietic cell-line (32D), p185 had less transforming activity than EGFr, suggesting that the two receptors are linked with different efficiencies to one or more signaling pathways [Di Fiore *et al.*, 1987a; Di Fiore *et al.*, 1987b; Difiore *et al.*, 1990b). By creating chimeric molecules with different tyrosine kinase or carboxyl-terminal domains, Di Fiore and colleagues (Di Fiore *et al.*, 1990b) demonstrated that the tyrosine kinase domain of either receptor was responsible for the specificity of mitogenic signaling in the 32D cells. The specific determinants of this mitogenic specificity were localized to the juxtamembrane region within the amino-terminal half of either receptor's kinase domain (Segatto *et al.*, 1991; Di Fiore *et al.*, 1992).

The carboxyl-termini of the receptor tyrosine kinases appear to modulate the intrinsic tyrosine kinase activities rather than substrate specificity. Deletion of the carboxyl-terminal 204 amino acids led to a 40-fold reduction in p185 transforming activity (Di Fiore *et al.*, 1990a), in agreement with the deletion and tyrosine mutations described above.

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When the carboxyl-terminal domain of p185 was substituted into the EGFr, tyrosine kinase and transforming activity were elevated.

These studies indicate that p185 and EGFr have intrinsically different catalytic activities as well as different substrate specificities which lead to disparate mitogenic effects. These observations may help to explain tissue-specific alterations of different oncogenic growth factor receptors in different human tumors (Di Fiore *et al.*, 1990b). Moreover, the aberrant coupling of two separate signaling pathways may also be involved in the synergistic transformation resulting from simultaneous expression of both receptor tyrosine kinases (Kokai *et al.*, 1989) as described below. Table 1 lists the proteins which are currently known to be associated with p185 or are phosphorylated by p185.

Another strategy to examine the signaling specificity of p185 is to catalog the physiological substrates for p185 and compare these proteins to EGFr-specific substrates. Scott and co-workers (Scott *et al.*, 1991) demonstrated that the treatment of breast-cancer cell-lines which overexpress p185 with an agonistic monoclonal antibody 4D5 stimulated p185 kinase activity and, subsequently, PI-4'-kinase and PI-3'-kinase activities and expression of the 'immediate early'

gene *c-fos*. Autophosphorylation of p185 and phosphorylation of a 56 kDa protein was also induced by mAb treatment.

Pretreatment of cancer cell-lines with a tyrosine phosphatase inhibitor (sodium orthovanadate) enhanced the phosphotyrosine content of $p185^{c\text{-}erbB\text{-}2}$ and the cytosolic proteins PLCγ and GTPase activating protein (GAP) in addition to several unidentified proteins (Jallal *et al.*, 1992). These putative p185 substrates were weakly associated with p185 and were, themselves, composed of a complex of phosphotyrosine containing proteins.

Further analysis of proteins associated with p185 was illustrated by Fazioli et al. (Fazioli *et al.*, 1991) who demonstrated a physical interaction between the human $p185^{c\text{-}erbB\text{-}2}$ and PLCγ and tyrosine phosphorylation of PLCγ and GAP by $p185^{c\text{-}erbB2}$. Again, using a chimeric (EGFR/erbB-2) protein construct, ligand induced activation of p185 resulted in the tyrosine phosphorylation of PLCγ, and the physical association between PLCγ and the cytoplasmic domain of p185 was also demonstrated. A p185 mutatant lacking 5 autophosphorlation sites was still able to phophorylate PLCγ although the affinity for this substrate was lower than in the wild type p185 (Segatto et al., 1992 Oncogene). $p185^{c\text{-}erbB\text{-}2}$ was also shown to tyrosine phosphorylate another cellular signaling substrate (GAP) at extremely low levels. The authors observed, however, that p185-mediated phosphorylation of GAP or PLCγ did not qualitatively or quantitiatively differ from that by EGFr, indicating that the increased mitogenic potency of p185 was not due to the utilization of these substrates (Fazioli *et al.*, 1991).

Peles and colleagues (Peles *et al.*, 1991) demonstrated the physical interaction of the activated $p185^{neu}$ (Glu664) and (PLCγ) through co-immunopreciptation of a 185kDa protein using an anti-PLCγ antibody in fibroblasts. This protein was identified as p185 by western blotting. The functional linkage of the two proteins was indicated by the tyrosine phosphorylation of immunoprecipitated PLCγ in cells expressing constitutively active p185, however, cells expressing normal p185 did not phosphorylate PLCγ. Additionally, the interaction of PLCγ with p185 was dependent on the tyrosine kinase activity of the receptor since introduction of a mutation at the ATP-binding site of transforming p185 (Ala758) abolished the kinase activity of the protein and disrupted p185/PLCγ interaction. Tyrosine phosphorylation of PLCγ was also lost in cells expressing the kinase deficient mutant. A p185-EGFR chimeric receptor was used to examine the effects of ligand stimulation of the proto-oncogenic $p185^{c\text{-}neu}$. EGF stimulation of the cells prior to immunoprecipitation resulted in p185/PLCγ interaction. These chimeric experiments indicate the p185 cytosolic domain can physically associate with PLCγ and p185 can phosphorylate tyrosine residues in this protein potentially leading to the activation of this signal transduction pathway.

The cytosolic PI-3'-kinase was also found to be a physiological substrate of p185 again using the chimeric EGFr/p185 approach (Peles *et al.*, 1992). EGF binding to the chimeric molecule led to the formation of a tyrosine phosphorylated complex between the p85 regulatory subunit of PI-3'-kinase and p185. PI-3'-kinase activity was inducibly enhanced and associated with p185. These authors also demonstrated that the lipid kinase activity was coupled with the full-length oncogenic rat p185 protein, but not a kinase inactive p185 mutant or a p185 protein lacking the carboxyl-terminal 255 amino acids.

The *shc* gene products are adaptor proteins which contain SH2 domains likely to be involved in the coupling of the *ras*-signaling pathway to activated growth factor receptors. Segatto *et al.* (Segatto *et al.*, 1993) demonstrated tyrosine phosphorylation of three shc polypeptides by the p185 kinase. Each of the 3 Shc isoforms could be co-immunoprecipitated with the wild type $p185^{c\text{-}erbB\text{-}2}$. This physical association was mediated by a direct interaction with the Shc SH2 domain as measured by *in vitro* reconstitution experiments. The carboxyl-terminal 179 amino acids and specific autophosphorylated tyrosine residues within this region were required for the physical association of Shc/p185 and phosphorylation of Shc by p185.

The *c-src* tyrosine kinase has also been implicated in p185 signal transduction (Muthuswamy *et al.*, 1994) by the demonstration of elevated c-src activity in mammary carcinomas induced by a MMTV/neu transgene. A recombinant *c-src* SH2 fusion protein was expressed and used to affinity purify p185 from protein extracts derived from neu-expressing tumors. The physical association of *c-src* and p185 *in vivo* was also confirmed by co-immunoprecipitation from a mammary tumor cell line, however, direct phosphorylation of *c-src* by p185 was not demonstrated (Muthuswamy *et al.*, 1994). The association of the activated $p185^{c\text{-}erbB\text{-}2}$ and *c-src* has also been demonstrated by Luttrell *et al.* (Luttrell *et al.*, 1994) in SK-BR-3 cells.

Protein tyrosine phosphatases may also play a role in the p185-mediated signal transduction pathway. Vogel et al showed the ability of a chimeric protein HER1-2 (EGFR fused to cytoplasmic domain of HER2-neu) to interact with 2 different SH2-containing phosphatases (Vogel *et al.*, 1993). Both phosphatases (PTP 1C or PTP 1D) were able to co-immunoprecipitate with this chimera. Co-expression of the EGFr/p185 and PTP 1C or PTP 1D followed by ligand (EGF) stimulation, cell lysis, and immunoblotting with anti-phosphotyrosine antisera revealed that expression of PTP 1C led to partial dephosphorylation of the chimeric receptor. However, expression of PTP 1D had no effect on dephosphorylation of the receptor. It is presently unclear as to whether these phosphatases are themselves substrates of the p185 tyrosine kinase and whether the phosphatase acts on p185 autophosphorylation or cytosolic substrate phosphorylation.

Physical and functional interaction of EGFr and p185

Numerous observations have indicated that p185 was activated in an EGFR-dependent manner (King *et al.*, 1988; Kokai *et al.*, 1988; Stern and Kamps, 1988). These observations suggested that p185 was indirectly activated by EGFr or could directly serve as a substrate for the EGFR tyrosine kinase resulting in p185 kinase activation. Since EGF does not bind to p185, these results suggested that EGF, acting through EGFr, can regulate the intrinsic kinase activity of p185 and EGF-induced phosphorylation of p185 in both normal and transformed cell lines.

The transregulation of EGFR and p185 has profound biological conseqences. A critical level of overexpression of either receptor protein was necessary to achieve a significant growth advantages and transformation *in vitro* (as described above). NIH 3T3 cells or NR6 cells which express $p185^{c\text{-}neu}$ alone at a moderately high level (10^5 receptors/cell) were not transformed (Hung *et al.*, 1989; Kokai *et al.*, 1989), unless EGFR was co-expressed at an equivalent level (Kokai *et al.*, 1989). Down-regulation of EGFr or $p185^{c\text{-}neu}$ from the cell surface by anti-receptor

antibody treatment reversed the transformed phenotype (Wada *et al.*, 1990). These results suggested that two distinct, moderately-overexpressed tyrosine kinases can synergistically interact leading to cellular transformation.

This hypothesis was supported by the direct evidence of the physical interaction of p185$^{c\text{-}neu}$ and EGFR (Wada *et al.*, 1990). Heterodimers of p185$^{c\text{-}neu}$ and EGFr were also detected in the absence of chemical cross-linkers, and non-covalent forces were predominantly involved in hetero-receptor association (Qian *et al.*, 1992). Heterodimeric association of EGFr and p185$^{c\text{-}erbB2}$ have also been found in the human breast cancer cell line SK-BR-3 (Goldman *et al.*, 1990) and transfected cells (Spivak-Kroizman *et al.*, 1992). Ligand binding to the EGFr stimulates the formation of heterodimers (Wada *et al.*, 1990; Qian *et al.*, 1992). Cytoplasmic domain deletion mutants of either EGFr or p185$^{c\text{-}neu}$ were still able to associate with full-length p185$^{c\text{-}erbB\text{-}2}$ (Spivak-Kroizman *et al.*, 1992) or EGFR (Qian *et al.*, 1994b), respectively. However, the association of EGFR with a mutant of p185 lacking the extracellular domain was undetectable (Qian, unpublished data). These data indicated that the extracellular domain is required for heterodimerization in a similar manner to the homodimerization of either protein (Lax et al., 1991; Spivak-Kroizman *et al.*, 1992). Furthermore, the predominance of the heterodimer of EGFr and p185 revealed that heterodimerization is favored over either form of homodimerization (Qian *et al.*, 1994b).

The functional activation of the receptor tyrosine kinase resulting from an intermolecular receptor-receptor mechanism is often followed by rapid trans-phosphorylation events. This has been observed with a number of protein tyrosine kinases, such as pp60$^{c\text{-}src}$ (Cooper and MacAuley, 1988), the insulin receptor (Boni-Schnetzler *et al.*, 1988), PDGFr (Kelly *et al.*, 1991), EGFr (Honegger *et al.*, 1990), and p185 (Qian *et al.*, 1994b). Trans-phosphorylation of EGFr and p185$^{c\text{-}neu}$ containing a mutation inactivating kinase activity has been observed in both transiently- (Connelly and Stern, 1990) and stably- tranfected cells (Qian *et al.*, 1994b). It appears that intermolecular phosphorylation of these two receptor species occurs reciprocally, as a kinase deficient EGFr (K721A) can also be phosphorylated by p185 proteins (Spivak-Kroizman *et al.*, 1992). An analysis of *in vivo* tyrosine phosphorylation and *in vitro* kinase acitivities indicated that the heterodimeric complex was more highly active than the p185 homodimer or the EGFr homodimer (Qian *et al.*, 1992). Recent studies have demonstrated that the heterodimer of EGFr and a mutant p185 lacking the cytoplasmic domain resulted in an inactive kinase complex (Qian *et al.*, 1994a; Qian *et al.*, 1994b). Therefore, these data suggest that the protein-protein interaction of the cytoplasmic domains of the two receptors was required for EGFr activation, and that the associated p185 protein is not simply a substrate for EGFr, but is also a transactivator for the EGFr kinase.

The intermolecular association and resultant tyrosine kinase activation between EGFr and p185$^{c\text{-}neu}$ modulated EGFr functions, including an increased EGF binding affinity and elevated sensitivity to EGF in mitogenic signaling (Wada *et al.*, 1990; Qian *et al.*, 1994a). Down-modulation of the p185 receptor by anti-p185 antibody resulted in the loss of the very high EGF-binding subclass, supporting the notion that heterodimerization was related to the increase of EGF affinity (Wada *et al.*, 1990). This effect of p185 on the ligand binding affinity of the homologous EGFr may also extend to the ligand binding of other EGFr family members including p160$^{c\text{-}erbB\text{-}3}$ or p180$^{c\text{-}erbB\text{-}4}$. These receptor molecules are likely to heterodimerize as well as form homodimeric structures

The heterodimerization of a kinase-deficient p185 with EGFr also caused inducible dominant negative suppression of normal EGFR functions. The unproductive heterodimer comprised of wild type EGFr and kinase inactive p185 impaired synergistic hetero-receptor signaling, suppressed the function of normal EGFR, and abolished the transformed phenotype in living cells, supporting a transregulation hypothesis of EGFr and p185 (Qian *et al.*, 1994a).

Numerous studies suggest the requirement of tyrosine kinase activity for a maximal rate of ligand-mediated receptor degradation as a general phenomena for growth factor receptor endocytosis (Honegger *et al.*, 1987; Glenney *et al.*, 1988; Downing *et al.*, 1989). EGF-induced EGFR degradation and p185 co-degradation in transfected cells (Qian *et al.*, 1994a) or in normal mammary epithelial cells (Kornilova *et al.*, 1992) provided further evidence that the heterodimer was an active kinase complex required for celllular processes. In contrast, receptor degradation was retarded in cells co-expressing EGFR and kinase deficient p185 proteins (Qian *et al.*, 1994a).

The mechanism of the synergistic interaction of the p185 and EGFr receptor tyrosine kinases has not been completely elucidated. Activation of two types of receptor kinases simultaneously may result in more efficient or different signal transduction pathways. Intermolecular association and trans-phosphorylation of receptor molecules result in an activated heterodimeric kinase which may provide qualitative or quantitiative differences in phosphotyrosine sites necessary for cellular substrate binding. Currently, specific substrates of EGFR and p185Neu/c-erbB2 kinases have not been identified. A carboxyl terminal-deleted p185 and EGFR can still associate into an active complex, but the kinase is insufficiently active to mediate a synergistic effect related to cell transformation (Qian *et al.*, manuscript in preparation). These data indicate the critical function of the carboxyl termini of either receptor, and provide an experimental method to examine substrates important for transformation. We propose that related members of the EGFr family are each capable of oligomerization in homo- or hetero- specific manners, creating higher order oligomeric structures with complex regulatory interactions.

The experimental model of p185 and EGFr interaction suggests a causal relationship between heterodimeric kinase activities and malignant transformation which may have clinical implications. The role of the EGFr and the p185$^{c\text{-}erbB\text{-}2}$ protein in breast cancer has been well documented (Harris *et al.*, 1989; Gullick, 1990). There are additional examples of human malignancies which may involve the aberrant expression of both EGFr and p185 (Dougall *et al.*, 1993) which will have additional prognostic value. These examples include the overexpression of EGFr and *c-erbB-2* in a number of tumors of epithelial origin, e.g. thyroid tumors (Aasland *et al.*, 1988), pancreatic tumors (Korc *et al.*, 1986; Cohen *et al.*, 1989a), or endometrical carcinomas (Wang *et al.*, 1993). These observations suggest that EGF and other ligands for EGFR and p185$^{c\text{-}erbB\text{-}2}$proteins may contribute to the development and maintenance of the malignant phenotype of these tumors. Moreover, other putative growth factor receptors such as p160$^{c\text{-}erbB\text{-}3}$ and p180$^{c\text{-}erbB\text{-}4}$ appear also to independently contribute to the development of certain malignancies (Kraus *et al.*, 1989; Plowman *et al.*, 1993).

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Proto-oncogenic $p185^{neu/c\text{-}erbB2}$ in developing and normal tissues

Much of our understanding of the physiological function of p185 is derived from observations in transformed cells. However, relatively little is known about its role in normal cell growth regulation and tissue development. Several observations have implicated p185 in mammalian development. Receptor tyrosine kinases, such as *c-ros*, *c-met* and *c-neu* are commonly expressed in embryonal epithelial cells. Interestingly, increasing evidence suggests that ligands for these receptors may be, or have been, found in mesenchymal tissues (reviewed in Birchmeier *et al.*, 1993). During development, mesenchymal cells characteristically produce signals which are necessary for differentiation and morphogenesis of epitheleal cells, and tyrosine kinase receptors and their ligands may be significantly involved in this interaction. Expression of DER, a tyrosine kinase receptor in *Drosophilia* and related to EGFr and p185 was shown to be developmentally regulated. Katzen *et al.* (Katzen *et al.*, 1991), demonstrated that mutations affecting DER expression in *Drosophilia* correlated with defective development of the nervous tissue, optic disc, ovary, and mouthparts.

Several studies have been performed to determine tissue specific expression of p185 in normal mammalian fetal and adult tissues (Table 2). Some inconsistent findings can be attributed to differences in methods of analyses, tissue preparation, and antibodies used. Kokai *et al.* (Kokai *et al.*, 1987) have shown that $p185^{c\text{-}neu}$ is expressed in a stage- and tissue- specific manner in the developing rat. Their results revealed that significant $p185^{c\text{-}neu}$ expression in the CNS was transient, from embryonic day number 14 to 16, which also corresponds to the period of highest susceptibility of the rat CNS to ethylnitrosourea induced neuroblastomas. These observations also implicated the involvment of p185 in mammalian neural development. Analysis of 11 human fetuses, 6-12 weeks of age, by immunocytochemistry also revealed expression of p185 in the peripheral and central nervous system (Quirke *et al.*, 1989). Staining was strongest within the neural processes, and the ependymal and marginal layers of the developing brain. However, immunostaining of the human fetal CNS was not seen in two other similiar studies. Evidence of significant p185 expression in the adult brain is lacking (Natali *et al.*, 1990; Press *et al.*, 1990), although our laboratory has recently identified expression of p185 in the adult brain.

The relationship of p185 expression in developmental regulation was also studied in the rat peripheral nervous system (Cohen *et al.*, 1992). Northern blot and immunohistochemical analysis of the sciatic nerves from rats revealed that Schwann cells expressed p185 from postnatal day 1 to 7. Interestingly, p185 expression was revived the first day after sciatic nerve transection and continuing for up to day 21. When Schwann cells grown in culture were stimulated with mitogens, forskolin and GGF, expression of p185 was increased. These results may imply that p185 expression in the Schwann cell is increased during nerve development and Wallarian degeneration, but axonal contact downregulates the receptors expression.

In rats and humans, studies revealed that expression of p185in the kidney was highest in fetal tissues and persisted at lower levels throughout adulthood (Table 2). Press (Press *et al.*, 1990) noted that immunohistochemical staining for p185 was weak in Bowman's capsule and proximal tubules and moderate in the collecting tubules of human fetal kidneys. This is in contrast to findings in the rat in which the proximal tubules were prominently stained (Kokai *et al.*, 1987; Gullick *et al.*, 1987).

p185 expression has also been reported in the epithelia of fetal human stomach (Press *et al.*, 1990) and fetal rat and human intestines (Cohen *et al.*, 1989b; Quirke *et al.*, 1989; Press *et al.*, 1990; Natali *et al.*, 1990). Expression continued on into adulthood in these tissues. Cohen, *et al.* (Cohen *et al.*, 1989b) noted that p185 specific staining was localized to epithelial cell at villus tips and not in the crypts of the intestines. Characterization of p185 expression in the fetal and adult liver has been inconclusive at this time (Table 2) Similar to those results found in the kidney, also appears to be expressed in rat and human fetal lungs with persistance of expression at lower levels throughout adulthood.

The human fetal epithelium of the female reproductive tract was positive for p185 expression and the adult vaginal epithelium also stained positively (Press *et al.*, 1990). Additionally, expression levels of the vaginal epithelium did not significantly fluctuate through out the menstral cycle (Press *et al.*, 1990) . Follicular cells of the ovary were positive, whereas there was no evidence of p185 in the oocytes. Quirke *et al.* (Quirke *et al.*, 1989) found that the human placenta had significant levels of receptor expression during all trimesters, however, p185 expression was found only in the last trimester of two other studies (Coussens *et al.*, 1985; Natali *et al.*, 1990).

The results of these studies imply that p185 has specialized functions in developmental and growth regulation of normal cells. The most consistent finding of these reports is that p185 was most commonly expressed in epithelial cells in the adult. Given that other tyrosine kinase receptors expressed in epithelial cells are involved in cellular differentiation and morphogenesis, it is not unlikely that p185 may have a similar role (Birchmeier *et al.*, 1993). Additionally, several of these studies have shown that p185 is expressed in different tissues and independent of EGFr (Gullick *et al.*, 1987; Mori *et al.*, 1987; Quirke *et al.*, 1989; Cohen *et al.*, 1992). Furthermore, the localization of p185 in different tissues further supports its assignment to specialized function. In the renal tubule epithelium, the receptor has been localized to the apical surface of the cell, whereas in the mullerian ducts, female reproductive tract, and gastro-intestinal epithelium p185 was localized to the lateral and basal cell membranes.

Interestingly, while p185 expression has been observed in the more mature cells of the intestinal villus, EGFr expressoin may be more limited to the immature cell types of the villus. A dramatic inverse distribution relationship of the EGFr and p185 in normal secretory epithelial cells was also observed in our studies of the skin (Maguire *et al.*, 1989) and in other tissues. In renal tissues an inverse relationship of the receptors was also noted.

The changing pattern and complex relationship of EGFr and p185 expression are seen in the dynamically changing genital tract. In the exocervix, p185 was most prominent in the intermediate layer and negative in the basal cells. However, EGFr was expressed prominently in the basal cells and minimally in the intermediate layers (Wang *et al.*, 1993). These studies support our original contention (Maguire and Greene, 1989) that these receptors are expressed in a defined manner that is relevant to the normal differentiation of epithelia. One possibility is that a genetically programmed and controlled alteration in p185 expression is critical for differentiation of epithelial tissues. Alterations in some level of this receptor distribution program of expression might lead to abnormalities of cell growth. Further elucidation of the ligand for p185 and the

Table 2. Expression of p185 in fetal and adult tis

Tissue (organism)	*fetal*	*adult*	*reference*
central nervous ystem	+	-	a,b,c,d,e
peripheral nervous system & vertebral column	+	N.D.	c
cartilage & bone	+/-	N.D.	c,e
muscle	+/-	-	c,e
skin	+*	+*	a,b,c,d,e,f
heart	+/-	-	c,d,e
lungs	+	+	a,b,d
gastro-intestines	+	+	a,b,c,d,e,g
kidney	+	+#	a,b,d,e,f,g
liver	-	-	d,e,g

N.D., not determined; +/- , variable results by different laboratories; * one study (Natali *et al.*, 1990) did not detect p185 in adult or fetal tissues; # one study did not detect p185 in adult kidney (Mori *et al.*, 1987); [a] Coussens *et al.*, 1985; [b] Kokai *et al.*, 1987; [c] Quirke *et al.*, 1989; [d] Press *et al.*, 1990; [e] Natali *et al.*, 1990; [f] Gullick *et al.*, 1987; [g] Mori *et al.*, 1987.

development of murine p185 knock-out models may be of great benefit in further defining the role of this receptor in normal tissues.

Ligands for p185

p185$^{c\text{-}erbB2}$ receptors have unusually complex activation pathways since they can form homodimeric and heterodimeric associations. Ligands which bind to other receptors (such as EGF) are thus able to activate p185 through heteroreceptor interactions, however, a ligand which binds directly to and activates p185 has proved elusive.

A number of recent reports have described the purification of biologically active ligands from various sources which are alternatively-spliced products of a single gene, termed heregulin or neu differentiation factor (Mudge, 1993). Each of these proteins were shown to induce activation of p185 tyrosine kinase acitvity, however, direct binding of these factors to p185 was not demonstrated. The heregulin gene products apparently bind to closely associated proteins which are likely to be p160$^{c\text{-}erbB\text{-}3}$ and p180$^{c\text{-}erbB\text{-}4}$ (Culouscou *et al.*, 1993; Yarden, 1994), but not to the p185 receptor itself (Peles *et al.*, 1993).

The activation of p185 by these various ligands through different receptors demonstrates the importance of oligomeric hetero-receptor assemblies on the cell surface. This trans-activation of p185 also illustrates the physiological importance of p185 in various developmental and differentiation processes. The neu differentiation factor (NDF) was identified as a 44 kDa glycoprotein secreted by *ras*-transformed Rat1-EJ cells. NDF was initially thought to be a ligand for p185 by virtue of p185 kinase activation (Peles *et al.*, 1992; Wen *et al.*, 1992), however direct binding of NDF to p185$^{c\text{-}neu}$ receptors could not be demonstrated (Peles *et al.*, 1993). NDF induced cell polyploidy, inhibited cell growth, and arrested the cell cycle at the late S or G2/M phases. This, and a related factor (gp30), induced the differentiation of mammary cells into milk-producing cells (Lupu *et al.*, 1990; Bacus *et al.*, 1992; Peles *et al.*, 1992; Wen *et al.*, 1992). Another related factor, heregulin, (45 kDa) has been reported to be produced by the MDA-MB-231 human breast carcinoma cell line (Holmes *et al.*, 1992), and may be a breast tumor mitogen illustrating the potential importance of autocrine growth loops.

In addition, two growth factors involved in neuorobiological processes may also signal through p185 pathways. The glial growth factors (GGFs) (Marchionni *et al.*, 1993) and ARIA (acetylcholine receptor inducing activity) (Falls *et al.*, 1993) have been purified and shown to activate the tyrosine kinase activity of a protein of 185 kDa. The NDF, GGFs, ARIA and heregulin molecules have been cloned and sequenced and are expressed by the same gene

In addition to the heregulin family of ligands which do not bind the p185 receptor directly, other factors have been described which activate p185 and may represent true ligands. A factor of 25 kDa has been isolated from bovine kidney and was mitogenic for DHFR-G8 and A431 cells (Huang and Huang, 1992). Our laboratory has isolated and characterized neu activating factor (NAF) from a transformed human T cell line, ATL-2, (Davis *et al.*, 1991; Dobashi *et al.*, 1991). NAF increased *in vitro* and *in vivo* tyrosine phosphorylation, receptor dimerization, and growth only in cell lines expressing p185 receptors. NAF also demonstrated unique properties from the ligands described above as illustrated by its specific binding and activation of p185 receptor purified from an baculovirus expression system and its distinct molecular mass (15-17 kDa) (Samanta *et al.*, 1994).

Reversion of the malignant phenotype of *neu*-driven tumors by anti-receptor antibodies

Antibodies reactive with the extracellular domain of p185 can reverse the phenotype of transformed cells. We showed that anti-p185 antibodies can rapidly down-modulate the receptor from the cell surface (Drebin *et al.*, 1985). The removal of the transforming receptor from the cell surface *in vitro* was associated with a reduction in the malignant phenotype and a conversion of the cellular phenotype to into a more normal one. *In vivo* studies then showed that administration of anti-receptor antibodies could cause retaration of tumor growth (Drebin *et al.*, 1986). Studies using mice which had been treated to eliminate the role of complement or macrophages defined that the effect of the antibodies was direct and related to

receptor down-moulation (Drebin *et al.*, 1988b).

In other experiments, our laboratory evaluated the activities of anti-receptor antibodies reactive with distinct domains of the p185 protein an established that down-moulation was even more effective as a consequence of the synergistic activity of separate antibodies (Drebin *et al.*, 1988a). *In vivo* the use of two distinct antibodies had the effect of completely arresting tumor growth and curing up to 60% of the animals with tumors (Drebin *et al.*, 1988a).

Our laboratory has also developed a transgenic model to study the preventative effects of anti-receptor antibodies on incipient tumor development. In the transgenic model, animals which express the oncogenic *neu* cDNA under the control of the MMTV promoter (Bouchard *et al.*, 1989) which develop tumors at about 30-35 weeks of age were used. Studies to date indicate that the onset of tumor appearance can be delayed until 50 weeks by the administration of as little as 10 µg weekly of anti-p185 antibody. We have been able to entirely prevent tumor appearance in the transgenics by doubling the amount of anti-p185 antibody administered (Katsumata, Okudaira, and Greene, in preparation).

Tumors resulting from the synergistic activities of p185 and EGFr (as described above) can also be treated *in vivo* with antibodies to either receptor to cause tumor elimination (Wada *et al.*, 1989). The observation that anti-receptor antibodies could revert the malignant phenotype of *neu* and *c-neu*/EGFr transformed cells *in vitro* and *in vivo* (Drebin *et al.*, 1985; Drebin *et al.*, 1986; Wada *et al.*, 1989), has been widely reproduced and represents a logical approach to specific anti-tumor therapy which has been taken to the clinic (Maier *et al.*, 1991; Shepard *et al.*, 1991; Harwerth *et al.*, 1993; Kasprzyk *et al.*, 1992). It has been suggested that there might be some benefit in using anti-p185 antibodies for the treatment of human breast cancer alone or in combination with other therapeutics. Collectively, these data support the concept that antibodies to the ectodomain of these receptors can be effective therapies *in vivo*. Members of this receptor family are commonly found together on cell surfaces and appear to contribute to the malignant features of neoplasia.

Consequently, the paradigm that two antibodies can synergistically affect transformation relates to the basic mode of operation of these receptors, namely that they operate as homo- or hetero-dimeric forms. Our data indicates that it may be more efficient to target two separate domains of these receptor forms than relying on the specificity of a single antibody. This can be achieved by two antibodies reactive with distinct regions, either on the same molecule or on either of the receptor monomers, or creating a novel bifunctional species of antibody that simultaneously binds both recpetor monomers.

A general synthetic strategy has been used to develop organic CDR structures which lack peptide bonded amino acids, are poorly immunogenic, are resistant to peptidases and which are non-toxic (Saragovi *et al.*, 1991; Saragovi and Greene, 1992). These mimetics behave in an identical biological manner to the immunoglobulin or CD4 proteins from which they were modeled. Their affinity of interaction with the respective ligands is in the low micromolar range (~4 µM). Recently, we have developed the technology to link 2 CDRs together and these complex molecules have binding affinities of 40-100 nM. We hope to use these molecules to treat *c-erbB2*-driven tumors in the near future.

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EXHIBIT D

EXHIBIT D

US005705157A

United States Patent [19]

Greene

[11] Patent Number: 5,705,157

[45] Date of Patent: Jan. 6, 1998

[54] **METHODS OF TREATING CANCEROUS CELLS WITH ANTI-RECEPTOR ANTIBODIES**

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[21] Appl. No.: **347,018**

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[58] **Field of Search** 424/138.1, 143.1, 424/155.1; 530/387.7, 388.22, 388.8, 388.85; 435/240.27

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[57] **ABSTRACT**

The present invention provides methods and therapeutic compositions for treatment of mammalian tumors wherein the cells of such tumor express both epidermal growth factor receptor and p185neu, p185c-neu or a homologue of p185neu or p185c-neu and the epidermal growth factor. At least one antibody specific for epidermal growth factor receptor, monoclonal antibody 425, and at least one antibody specific for p185neu, p185c-neu or a homologue of p185neu or p185c-neu, monoclonal antibody 7.16.4, are administered to the mammal in an amount effective to reduce tumor growth.

6 Claims, 1 Drawing Sheet

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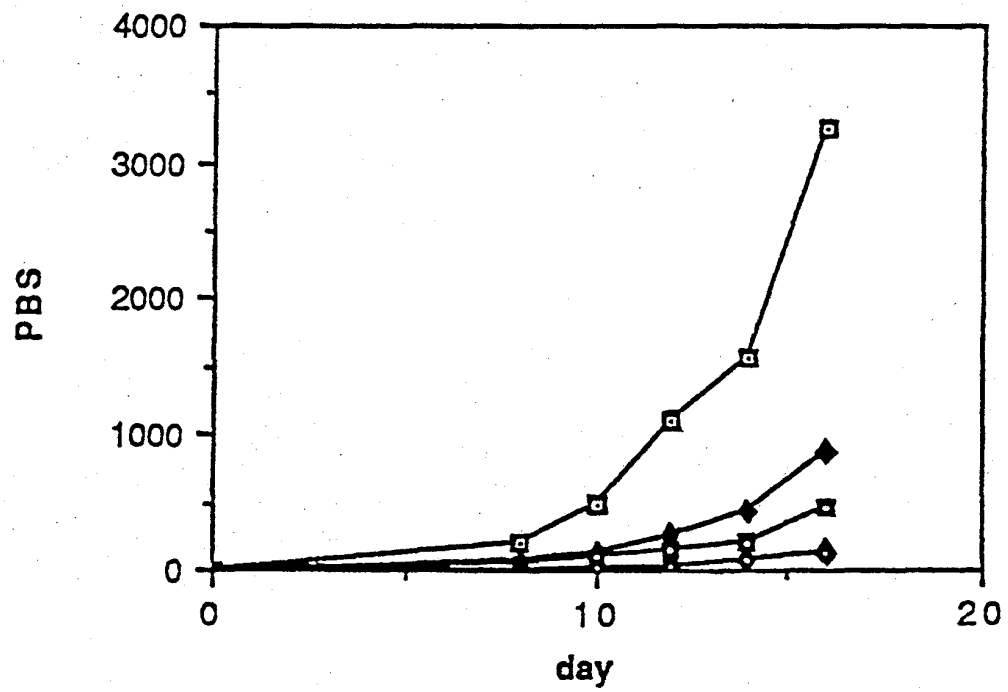
Figure 1



METHODS OF TREATING CANCEROUS CELLS WITH ANTI-RECEPTOR ANTIBODIES

This is a continuation of application Ser. No. 07/386,820, filed Jul. 27, 1989, now abandoned.

FIELD OF THE INVENTION

The present invention is related to the field of treatments of mammalian tumors. More particularly this invention relates to methods for treating mammalian tumors employing antibodies and novel therapeutic compositions for such treatment.

REFERENCE TO RELATED APPLICATIONS

This application is related to co-pending U.S. application Ser. No. 126,572 filed Nov. 30, 1987 in the names of Mark I. Greene and Jeffrey A. Drebin.

BACKGROUND OF THE INVENTION

Current tumor treatments rely for the most part in the cytotoxic effects of drugs and radiological therapy. Although these treatments bring remission and cure to some patients, they unfortunately have serious side effects because they kill not only tumor cells but also normal non-tumorous cells. There exists a great need for mammalian tumor treatments which affect primarily the tumor cells, but that have minimal interference with normal cells and cellular functions.

Recent studies in the molecular genetics of cancer indicate that certain genes known as oncogenes may play a role in the transformation of some cells from their normal condition to a cancerous condition. Proto-oncogenes, genes closely related to these genes, are found in somatic cells of all eukaryotic species examined and have been highly conserved in evolution and it is thought that proto-oncogenes normally play critical roles in cellular growth and development. Oncogene amplification and chromosomal rearrangements involving oncogenes have been detected in a large number of tumors. Furthermore, some tumors have been shown to contain activated oncogenes which, in DNA transfection assays, are capable of conferring neoplastic properties upon non-neoplastic rodent fibroblast cell lines. Collectively these studies suggest that alterations in proto-oncogene structure and function play a critical role in the development of neoplasia.

Although most oncogene-encoded proteins reside in the nucleus or the cytoplasm, some oncogenes encode proteins that express antigenic sites on the cell surface. For example, the erbB, fms and ros oncogene products are transmembrane glycoproteins that possess extracellular domains. The sis oncogene product may also exist in a membrane associated form on the surface of transformed cells. Another oncogene which encodes a protein that exposes antigenic sites on the surface of transformed cells has been identified by transfection of DNA from ethyl nitrosourea-induced rat neuroblastomas into NIH3T3 cells. This oncogene has been termed neu. The neu gene has been found to be amplified in some human tumors, particularly those of the breast, suggesting that this gene may play a role in the etiology of human cancer.

Amplification of c-erB-2, the human homologue of the rat c-neu gene, occurs with high frequency in some human adenocarcinomas of the breast, pancreas and ovary. Increasing c-erbB-2 expression levels may correlate with the clinical progression of adenocarcinoma of the breast. The neu oncogene and p185 have also been found active in human adenocarcinomas including breast, lung, salivary gland and kidney adenocarcinomas, as well as prostate neuroblastoma. In human primary breast cancers, amplification of the neu oncogene was found in about 30% of all malignant tumors examined. Increased malignancy, characterized by large tumor size and increased number of positive lymph nodes as well as reduced survival time and decreased time to relapse, was directly correlated with an increased level of amplification of the neu gene. The neu proto-oncogene is expressed at low levels in normal human tissues.

The neu oncogene was originally isolated from rat neuroblastomas that developed in the offspring of rodents exposed to ethylnitrosourea at a discrete time period of gestation. The neu oncogene encodes an 185 Kd surface glycoprotein, termed p185neu, that possesses tyrosine kinase activity, and is structurally similar to the EGF receptor at the nucleotide and amino acid level. However, p185neu has been shown to be distinct from the EGF receptor by detailed molecular analysis and chromosomal localization studies. The rodent cellular neu cDNA has been compared to the oncogenic neu cDNA, and the normal cellular gene product, p185c-neu, has been found to differ from the oncogene product by a single amino acid substitution (valine to glutamic acid) in the transmembrane anchoring domain. p185neu becomes activated by this point mutation in its transmembrane region. p185c-neu has been found in variety of tissues derived from developing and adult animals in a developmental stage and tissue specific manner. Both p185neu and its normal, cellular homologue, termed p185c-neu, possess tyrosine kinase activity, although the oncogenic form has greater, kinase activity. The neu encoded tyrosine kinase domain identifies the neu gene as a member of a large tyrosine kinase gene family. p185c-neu is highly homologous to, yet distinct from the epidermal growth factor receptor (EGFR).

The epidermal growth factor receptor protein is a structure of 170 kd mass that has been identified on both normal glial and glioma cells. Analysis of the EGF receptor's (EGFR) deduced primary structure suggests that is a transmembrane glycoprotein receptor which contains a tyrosine kinase domain. The extracellular portion of 621 amino acids of this receptor, which spans the membrane only once, makes up the ligand binding domain. By analysis of shared sequences, it has been determined that the EGF receptor is the proto-oncogene product of the transforming v-erbB oncogene. The v-erbB oncogene was first characterized from the avian erythroblastosis virus. The EGF receptor encoding gene is often found in amplified forms in glioblastoma. The EGF receptor is structurally and antigenically related to the neu oncogene encoded receptor. Despite structural similarities, neu and erbB also differ and are separated in the genome. The erbB gene has been mapped to human chromosome 6, whereas the neu gene resides on chromosome 17.

In in vitro studies, c-erbB-2 transfection and subsequent overexpression of p185c-erbB-2 results in transformation of NIH3T3m cells. Similar studies with overexpressed EGFR reveal EGF-dependent cell transformation in vitro assays, but these same cells are not tumorigenic in nude mice. Unlike the NIH3T3 erbB-2 transfectants, NIH3T3 cell lines expressing high levels of p185c-neu are not transformed.

EGF, in an EGFR dependent manner, stimulates normal and transformed rodent and human cell lines resulting in increasing tyrosine phosphorylation of p185neu, p185c-neu, and p185c-erbB-2 with a concomitant increase in their respective tyrosine kinase activities.

SUMMARY OF THE INVENTION

The invention provides novel methods for treating mammalian tumors expressing both the epidermal growth factor receptor and p185 neu, p185c-neu, or a homologue of p185 neu or p185c-neu, such as erbB-2 on the surface of tumor cells. At least one antibody specific for epidermal growth factor receptor and at least one antibody specific for p185 neu, p185c-neu or a homologue of p185 neu or p185c-neu are administered to the mammal in an amount effective to suppress tumor growth. The antibodies work synergistically to suppress tumor growth; the administration of a combination of antibodies having a greater effect in reducing tumor growth than the administration of either type of antibody alone. The antibodies are preferably specific for an external domain of the epidermal growth factor receptor and p185 neu, p185c-neu, or homologue of p185 neu or p185c-neu.

The invention also provides novel therapeutic compositions for treating mammalian cancer tumors having cells which express both the epidermal growth factor and p185 neu, p185c-neu or homologue of p185 neu or p185c-neu. The compositions of the invention comprise at least one antibody specific for p185 neu, p185c-neu or homologue of p185 neu or p185c-neu, at least one antibody specific for epidermal growth factor receptor and a pharmaceutically acceptable carrier or diluent.

It has been found that two distinct overexpressed tyrosine kineses, p185c-neu and EGFR can act synergistically to transform NIH 3T3 cells, thus identifying a novel mechanism that can lead to transformation of cells. Overexpression of p185c-neu alone in rodent fibroblasts does not result in a transformed cellular phenotype; nor does overexpression of EGFR alone. However, overexpression of both EGFR and p185c-neu in the same cell does result in cellular transformation.

Applicant has also demonstrated that administration of a mixture of antibodies specific for extracellular domains of EGFR and p185neu surprisingly have a synergistic effect in reducing or abolishing cancer tumor growth in mice. Administration of a mixture of both types of antibodies slow tumor growth to a much greater degree than administration of antibodies to either EGFR or p185neu alone.

In general, the antibodies used for tumor immunotherapy by passive transfer of monoclonal antibodies are specific against random structures on the malignant cell surface, rather that cell surface molecules required for maintenance of the transformed phenotype. Antibodies specific for structures that themselves are necessary for neoplastic cell function, as are EGFR and p185c-neu, represent a more potent and efficient approach to cancer treatment.

The methods of the invention are useful in treating mammalian cancer tumors expressing both EGFR and p185neu, p185c-neu, or a homologue of p185c-neu or p185neu, such as erbB-2, the human homologue. Human tumors often have elevated levels of c-erbB-2 and/or EGFR.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 shows a graph of the effects of monoclonal antibodies to EGFR and p185c-neu and a combination of these antibodies upon tumor growth.

DETAILED DESCRIPTION OF THE INVENTION

In the methods of the invention antibodies, preferably monoclonal antibodies, are administered to mammals having a tumor expressing both epidermal growth factor receptors (EGFR) and p185 neu, p185c-neu or homologue of p185 neu or p185c-neu on the cell surfaces in amount effective to reduce tumor size.

Antibodies to EGFR and p185 neu, p185c-neu or homologue of p185 neu or p185c-neu are preferably administered to the mammal in combination with a pharmaceutically acceptable diluent or carrier, such as a buffer or saline solution. The antibodies may be administered to the mammal mixed together in the same diluent or carrier as a single dose, or they may be administered to the mammal in separate diluent or carrier solutions as separate doses, at substantially the same time or at different times. The antibodies are preferably administered to the mammal by injection into the tumor or tissue near the tumor. Other suitable methods of administration include oral, intraperitoneal, intramuscular and other conventional routes of pharmaceutical administration. The antibodies are administered to the mammal for a length of time effective to reduce tumor size and as needed to maintain regression of the tumor.

Antibodies useful in the methods of the invention may be made by conventional methods of producing monoclonal or polyclonal antibodies, such as the method in Harlow and Lane, eds., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988. A general method for the production of monoclonal antibodies comprises the steps of immunizing an animal such as a mouse or rat with an antigen to which monoclonal antibodies are desired. After allowing time for the immune system to generate lymphocytes capable of producing antibodies to the antigen, the animal is sacrificed and a suspension of spleen cells is prepared. The spleen cells are then fused with myeloma cells by contacting them in the presence of a fusion promoter such as polyethylene glycol. A percentage of the cells fuse to produce hybridomas. The earlier immunization of the animal from which the spleen cells were removed results in a number of lymphocytes which secrete antibody to the antigen of interest, a characteristic that is transferred genetically to the hybridoma during fusion of the spleen and myeloma cells. Hybridomas secreting monoclonal antibody having the desired specificity are then isolated using routine screening techniques.

Antibodies suitable for use in the methods of the invention are preferably specific for extracellular domains of the receptor i.e., these portions of the molecule which extend beyond the transmembrane region into the extracellular region. The extracellular domains of the EGFR are amino acids 1 through 621, Ullrich et al. Nature 309: 418–425. The extracellular domains of p185c-neu are amino acids 1–657, Bargmann et al. (1986) Nature 319: 226–230. Antibodies to the extracellular domains of these receptors may be produced starting with purified receptor or cells expressing the receptor on their surfaces.

Purified EGFR for use in producing antibodies suitable for use in the methods of the invention may be obtained from cells expressing EGFR, such as placental tissue, tumors or cell lines expressing EGFR, such as A431 carcinoma cells, Ullrich et al. Nature 309: 418–425, or the cell lines described herein. Alternatively, cells expressing EGFR on their surface may be used as the starting material for production of antibodies. Suitable cells include the cell lines disclosed herein, tumor cells expressing EGFR and other cells expressing EGFR.

Monoclonal antibodies specific for extracellular domains of EGFR may be selected by screening for binding of the antibodies to cells expressing EGFR. Antibodies binding to EGFR expressed on the surface of cells will be specific for

extracellular domains of EGFR. The antibodies may be further screened by testing for binding of the antibodies to cells not expressing EGFR on their surface; no binding additionally indicating specificity for EGFR. Suitable methods for production and selection of monoclonal antibodies include the method of Richert et al. (1983) Journal of Biological Chemistry 258: 8902–8907.

p185c-neu or p185neu can be obtained from cells expressing the receptors for use in producing antibodies specific, or the cells expressing p185c-neu or p185neu may be used for producing antibodies. Suitable cells include those described herein. NIH 3T3 cells transformed by the neu gene, either the oncogenic or normal allele as described in Hung et al. (1986) Proc. Natl. Acad. Sci. USA 83: 261–264, Bargmann and Weinberg (1988) Proc. Natl. Acad. USA 85: 5394–5398, and Bargmann et al. (1986) Nature 319: 226–230.

Monoclonal antibodies specific for extracellular domains of p185c-neu or p185neu may be selected for by screening for binding of the antibodies to cells expressing p185c-neu or p185neu. Antibodies binding to p185c-neu or p185neu expressed on the surface of cells will be specific for extracellular domains of p185c-neu or p185neu. The antibodies may be further screened by testing for binding of the antibodies to cells not expressing p185c-neu or p185neu on their surface; no binding additionally indicating specificity for p185c-neu or p185neu. Suitable methods for production and selection of monoclonal antibodies include the method of Drebin et al. (1984) Nature 312: 545–548. Briefly, in this method monoclonal antibodies specific for extracellular domains of p185neu or p185c-neu may be selected for by initially screening culture supernatants from growing hybridomas for the presence of antibody capable of binding B104-1-1 cells by indirect immunofluorescence using fluorescence activated cell sorting (FACS). Positive supernatants may then be tested for specificity by determining whether they contain antibody capable of binding normal NIH 3T3 cells, or NIH 3T3 cells transformed by transfection with Harvey sarcoma virus proviral DNA (cell line XHT-1-1a).

Chimeric antibodies having a specificity for both the epidermal growth factor receptor and p185 neu, p185c-neu or a homologue of p185 neu or p185c-neu, such as erbB-2 combine specificity for two different antigens in the same molecule. Chimeric antibodies may be produced by fusing two separate monoclonal antibody producing cell lines, so that the resulting cells produce monoclonal antibodies having specificity for both antigens. Chimeric antibodies of this type may be made by procedures known in the art, such as the method in Milstein and Cuello (1984) Immunology Today 5: 299–304, the disclosures of which are specifically incorporated as if fully set forth herein.

Antibodies suitable for use in the methods of the invention may be conjugated to other types of molecules such as cytotoxic molecules to enhance the tumor-reducing properties of the antibodies or provide other means of treating the tumors. For instance, the antibodies may be conjugated with a radioactive molecule, or therapeutic molecule such as a drug or other cancer treatment.

It is within the scope of the invention to use fragments of antibodies specific for epidermal growth factor receptor, p185 neu, p185c-neu, or a homologue of p185 neu or p185c-neu that exhibit binding specificity to these receptors. It is also within the scope of the methods of the invention to use peptides bindable with epidermal growth factor receptor, p185 neu, p185c-neu, or a homologue of p185 neu or p185c-neu, such peptides comprising at least a portion of the hypervariable region of an antibody specific for epidermal growth factor receptor, p185 neu, p185c-neu, or a homologue of p185 neu or p185c-neu. These peptides may also be joined at one end by a means such as a disulfide bond to form peptide dimers having one peptide bindable with EGFR and one peptide bindable with p185 neu, p185c-neu, or a homologue of p185 neu or p185c-neu.

Experimental Procedures

Preparation of Monoclonal Antibodies Specific for p185neu

C_3H/HeJ mice were repeatedly immunized with NIH 3T3 transfectants transformed by the neu oncogene (cell line B104-1-1) emulsified in Freund's adjuvant. Spleens from immune mice were fused with the aminopterin-sensitive NS-1 myeloma line, and hybridomas were selected in hypoxanthine-aminopterin-thymidine media. Culture supernatants from growing hybridomas were initially screened for the presence of antibody capable of binding B104-1-1 cells by indirect immunofluorescence using fluorescence activated cell sorting (FACS). Positive supernatants were then tested for specificity by determining whether they contained antibody capable of binding normal NIH 3T3 cells, or NIH 3T3 cells transformed by transfection with Harvey sarcoma virus proviral DNA (cell line XHT-1-1a). Selection of antibodies was performed according to these methods as disclosed in Drebin et al. (1984) Nature 312: 545–548, the disclosures of which are specifically incorporated as if fully set forth herein. Five hybridomas were identified that produce monoclonal antibodies capable of specifically binding B104-1-1 cells. One of the hybridomas produces an antibody termed 7.16.4 which is an immunoglobulin of the IgG2a isotype. Antibody 7.16.4 appears to recognize a cell-surface antigenic determinant specifically expressed by cells containing the neu oncogene.

Cell Culture Techniques

Cell lines were routinely cultured in 175 mm tissue culture flasks (Corning), containing 10 ml of Dulbecco's modified Eagle's medium (DMEM<K.C. Biologicals) supplemented with 5% heat inactivated fetal calf serum, 1% pen-strep mixture (M.A. Bioproducts). Transformed cell lines were passaged twice weekly at a 1:20 dilution following release from the tissue culture dish surface with Trypsin-Versene (M.A., Bioproducts). Nontransformed NIH3T3w cells or NR6 cells were passaged in a similar fashion, but at higher dilution (1:30–1:50) to prevent the development of spontaneous transformants.

Transfection and Cell Isolation

DNA transfection into 1×10^6 cells was carried out by the calcium phosphate precipitation technique (Graham and Van der Eb (1973) Virology 52: 456–467. The plasmids used in this study were pSV2-neuN, prepared according to the method of Bargmann et al. (1986) Nature 319: 226–230 (contains DNA coding for p185-neu), pEGFR1 prepared according to the method of Gorman and Miyajima (1988) J. Cell Biochem. Supplement 12A. C219 (a gift from A. Miyajima, DNAX) (contains DNA coding for epidermal growth factor receptor), pSV2NEO (Southern and Berg, (1982) J. Mol. Appl. Gent. 1: 327–341, and pSV2DHFR (Hung et al. (Proc. Natl. Acad. Sci. USA 83: 261–264). Two days after transfection, the cells were split and grown in the presence of 600 μM methotrexate in Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum. pEGFR1, the human EGFR cDNA is cloned under the transcriptional control of the SV40 early promotor element and the R-U5 segment of human T cell leukemia virus type 1 LTR (Takebe et al., (1988) Mol. Cell. Biol. 8: 466–472). 1 μg of pEGFR1 was used for each transfection.

Anchorage-Independent Growth Assay

Anchorage-independent growth capability was determined by assessing the colony-forming efficiency of cells suspended in soft agar according to the method in Drebin, et al. (1985) Cell 41: 695–706. All experiments to determine colony-forming efficiency were conducted using 50 mm tissue culture dishes containing a 6 ml cell free feeder layer and a 1 ml top layer in which 1×10^3 or 1×10^4 cells were suspended. Colonies were counted at 21 days for all cell lines. Each experimental group represents the mean of triplicate of quadruplicate samples.

Experimental Animals

Nude mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and were housed in the Research Animal FAcility of the Department of Pathology, University of Pennsylvania School of Medicine. Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the University of Pennsylvania and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, national Research Council (DHEW publication NIH 78-23, revised 1978).

Phosphorylation and Immunoprecipitation

All reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise indicated. 1×10^6 cells were plated, cultured for 24 hours and then incubated with inorganic ^{32}P (Amersham) at 0.5 mCi/ml in 5% FCS/phosphate free RPMI for 16 hours. The cells then were incubated for 15 minutes at 37° C. with each ligand at various concentrations EGF was obtained from GIBCO). Cells were washed with cold phosphate buffered saline (PBS) containing 400 uM sodium othovanadate and they lysed in PI/RIPA buffer, (1% NP40, 1% Deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate pH 7.4, 1% Trasylol, 1 mM PMSF, 2 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 400 uM sodium othrovanadate, 10 mM iodoacetoamide and 1 mM ATP0 for 30 minutes. Pre-cleared supernatants were subjected to immunoprecipitation with 7.16.4, a monoclonal antibody specific for p185neu/p185c-neu or with M294, an EGFR-specific antibody (ICN Biomedicals). Antibody 7.16.4 was prepared according to the method of Drebin et al., (1984) Nature 312: 545–548.

Immune Complex Kinase Assay

3×10^6 Ml cells were incubated in DMEM/5%FCS with or without 10 ng/ml EGF for 15 minutes at 37° C. Cells were washed twice with cold PBS and scraped into 1 ml of lysis buffer (1% Triton X-100, 1% Deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01M Sodium pyrophosphate pH 7.4 1% Trasylol, 1 mM PMSF, 2 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 400 uM sodium orthovanadate, 10 mM iodoacetamide and 1 mM ATP) and incubated for 30 minutes. The lysates were triturated, then incubated for 10 minutes with 50 ul of 50% (v/v) Protein A-Sepharose and centrifuged for 5 minutes to preclear the lysates. Precleared supernatants were incubated with 5 μg of 7.16.4 for 30 minutes, followed by 50 ul of 50% (vol/vol) protein a-sepharose for 30 minutes with rotation. Immune complexes were collected by centrifugation and washed four times with 500 ul of washing buffer (0.1% Triton X-100, 0.4 mM EDTA 10 uM sodium fluoride, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 400 uM sodium orthovanadate, 0.01 sodium fluoride, pH 7.4) then twice with 750 ul reaction buffer (20 mM Hepes pH7.4, 3 mM $MnCl_2$, 30 uM Na3SO4, and 0.1% Triton X-100). Pellets were resuspended in 50 ul of reaction buffer and [Gamma ^{32}P]-ATP was added to yield a final concentration of 10 uM. The samples were incubated at 27° C. for 15 minutes and the reaction terminated by addition of 3X SDS-PAGE sample buffer pH 6.8 (3% SDS, 10% Glycerol, 5% 2-mercaptoethanol, 0.4% bromphenol blue) containing 2 mM ATP and 2 mM EDTA and incubated at 100° C. for 5 minutes before SDS-PAGE analysis above.

^{125}I-EGF and ^{125}I Anti-p185neu Mononclonal Antibody Binding Assays

Binding assays using lactoperoxidase iodinated EGF (43,000 cpm/ng) or highly purified antibody 7.16.4 (12,000 cpm/ng) were performed according to the method of Kokai et al. (1987) Proc. Natl Acad. Sci USA 84: 8498–8501. Data were analyzed on a Scatchard plot, and apparent K_D and B_{max} determined.

Scatchard analyses were performed to study the affinity states of EGF receptors on M1 and K2 cells. The binding profiles on M1 and K2 cells reveal that each clone has two different affinity states for EGF. About 8% of the receptors for EGF expressed by the clones were high affinity. The M1 and K2 K_D values for EGF binding were 1.6×10^{-11}M and 6.0×10^{-10}M respectively for high affinity receptors and 3.1×10^{-9}M and 9.7×10^{-8}M respectively for the low affinity receptors (see Table 1). Table 1 shows the transformation parameters of the cell lines and summarizes the transformed phenotypes of the transfectants. For plating efficiency in soft agar, 1×10^3 and 1×10^4 cells were seeded in normal medium containing 0.25% agarose and colonies were counted after fourteen days. Results are expressed as percentage of seeded cells. Receptor numbers were determined by ^{125}I-anti-p185c-neu binding assays as described in Experimental Procedures, the total number o receptors per well were divided by the total number of cells per well. A 1:1 EGF to EGFR and a 1:2 anti-p185c-neu stoichiometry was assumed. The Kd's were determined by Scatchard analysis. To test for in vivo growth potential, individual clones (10^6 per animal) were washed, resuspended in phosphate buffered saline, and injected subcutaneously into 30 to 40 day old nude mice. Results are expressed as the number of mice developing tumors/total number of animals injected. These data show that both p185c-neu and the EGF receptors on these cells exhibit all the expected physical and functional properties including molecular weight, phosphorylation profile, tyrosine kinase activity, and ligand binding affinity.

TABLE 1

	Parental		Soft agar	Tumor	Receptor numbers		
Cell Lines	Cell Lines	Morphology	(% efficiency)	Incidence	p185neu	EGFR	Kd of EGFR
NR6	—	normal	<0.1	0/6	0	0	—
NV	NR6	normal	<0.1	0/6	1.7×10^5	0	—
M1	NR6	transformed	0.2–1.1	6/6	1.3×10^5	1.4×10^5	1.6×10^{-11} 3.1×10^{-9}

TABLE 1-continued

Cell Lines	Parental Cell Lines	Morphology	Soft agar (% efficiency)	Tumor Incidence	Receptor numbers p185neu	Receptor numbers EGFR	Kd of EGFR
KS	NR6	transformed	0.2–1.1	6/6	1.8×10^5	6.8×10^5	6.0×10^{-10} 9.7×10^{-8}
NE19	NR6	normal	<0.1	0/6	0	2.5×10^5	nd
NIH3T3w	—	normal	<0.1	0/6	0	0	—
G8	NIH3T3w	normal	<0.1	0/6	3.4×10^5	0	—
GEr	NIH3T3w	transformed	1–1.2	6/6	2.0×10^5	4.6×10^4	nd
B104-1-1	NIH3T3w	transformed	37.5	6/6	$1.4 \times 10^{5*}$	0	—
Rat-1	—	normal	<0.1	6/6	1.9×10^4	2.8×10^4	nd
A431	—	—	nd	0/6	0	2.6×10^6	nd

Tumor Growth Suppression In Effector Cell Independent Conditions

To determine whether tumor growth suppression observed in vivo was caused by antibody-mediated host cell dependent effects, the ability of the anti-receptor antibodies to suppress the transformed phenotype of the M1 and K2 cells under in vitro, effector cell independent conditions was examined. A focus-formation assay was performed, as the efficiency of growth in soft agar of M1 and K2 cells is low (<2%).

Fifty cells from each cell line were plated in 6 cm culture dishes with 1×10^4 of NR6 cells and cultured in the DMEM containing 2% of fetal calf serum. Sixteen hours later the medium was replaced to 2% FCS-DMEM containing growth factors and antibodies as indicated on Table 2. The media was changed every 3–4 days and morphologically transformed foci were scored on day 14. Numbers represent mean ± SD. These data were confirmed by three distinct experiments and each experiment was performed in duplicate. ++ indicates more than one hundred thousand molecules of receptors are expressed per cell. + indicates ten thousand to one hundred thousand molecules of receptors are expressed per cell.

Treatment of M1 and K2 cells with micromolar concentrations of EGF results in decreased number of foci as well as a change in cell morphology to a flatter more well spread phenotype.

Overexpression of p185c-neu in Rodent Fibroblasts Does Not Result in Transformation

The transforming activity of p185c-neu in two independent rodent fibroblast cell lines, NIH3T3w and NR6 was examined. The NIH3T3w cell line is an NIH3T3 cell subclone which displays a low frequency of spontaneous tumor formation and which lacks EGF receptors. NR6 is an EGFR-negative mutant of the EGFR-positive Swiss 3T3 mouse fibroblast cell line. NR6 cells were cotransfected with pSV2-neuN, an expression vector containing the p185c-neu coding region under the control of an SV40 promotor (Bargmann et al., (1986 Nature 319: 226–230), and pSV2DHFR using the calcium-phosphate precipitation method and cells were cultured in media containing 600 μM methotrexate. After methotrexate selection, p185c-neu overexpressing cells were isolated and named NV cells. The transformed phenotype of G8 cells, which are NIH3T3w cells cotransfected with the c-neu gene and pSV2DHFR (Hung et al., (1986) Proc. Natl Acad. Sci. USA 83: 261–264) was also examined.

TABLE 2

Cell Line	Expression Level p185c-neu	Expression Level EGFR	Control	Monoclonal Antibodies 7.16.4 1 μg/ml	Monoclonal Antibodies 425 1 μg/ml	Monoclonal Antibodies 9BG5 1 μg/ml	Growth Factors EGF 1 μg/ml	Growth Factors EGF 1 μg/ml	Growth Factors Insulin 1 μg/ml
M1	++	++	21 ± 1.4	11 ± 2.1	13 ± 1.4	25 ± 3.5	12 ± 0.7	21 ± 2.1	19 ± 2.1
K2	++	+	23 ± 1.4	11 ± 2.1	10 ± 2.1	21 ± 2.8	11 ± 1.4	17 ± 2.8	20 ± 0.7
GEr	++	++	60 ± 1.4	27 ± 3.5	46 ± 2.1	nd	30 ± 5.6	58 ± 3.5	54 ± 5.7
Ha-ras transformed NIH3T3w	none	none	29 ± 2.1	27 ± 3.5	26 ± 2.1	nd	25 ± 1.4	28 ± 0.7	31 ± 4.9
NV	++	none	0	0	0	nd	0	0	0
G8	++	none	0	0	0	nd	0	0	0
NE19	none	++	0	0	0	nd	3 ± 0.7	1 ± 0	0
NIH3T3w	none	none	0	0	0	nd	0	0	0
NR6	none	none	0	0	0	nd	0	0	0

1 μg/ml of Mab 7.16.4 or Mab425 reduced the number of foci by 50%. In addition, the M1 and K2 foci were significantly smaller than the foci in control media. Neither Mab7.16.4 nor Mab425 antibody was capable of inhibiting focus formation of a ras-transformed cell line, and the isotype matched 9BG5 monoclonal antibody could not inhibit focus-formation of either the M1 or K2 cells. These data indicate that specific effects exerted on the two receptor proteins by monoclonal antibodies result in suppression of the transformed phenotype even in the absence of effector cells.

Transfectants expressing 2×10^5 to 4×10^5 copies of p185c-neu were assayed for the transformed phenotype. Seven independent transfections were performed and 29 clones were examined for morphology, focus formation, colony formation in soft agar, and tumor formation in nude mice. Although the expression level of p185c-neu in the transfectants was comparable to those seen in certain human adenocarcinomas, none of the clones tested appeared to be transformed (see Table 1).

Overexpression of EGFR Alone In Rodent Fibroblasts Does Not Result In Cellular Transformation

It has been shown that overexpression of EGFR transforms NIH3T3 cells only when EGF is added to the culture medium. Accordingly NR6 cells were transfected with an EGFR expression vector. NR6 clones which overexpressed EGFR, named NE19, were not tumorigenic and did not form foci or soft agar colonies in a medium containing 10% FCS (see Table 1). Thus, transfected EGFR alone was unable to fully transform NIH3T3w and NR6 cells. In summary, neither p185c-neu nor EGFR overexpression alone is sufficient to transform NIH3T3w or NR6 cell lines.

Transfection of EGFR Into Cells Overexpressing p185c-neu Results In Cellular Transformation

To study the interaction of the two tyrosine kinases (EGFR and p185c-neu), EGFR cDNA was transfected into cloned cell lines that already express high levels of the p185c-neu. The resultant cell lines, NVEr and GEr, overexpress both p185c-neu and EGFR. These double transfectants were fully transformed by the introduction of the EGFR. However, cells which overexpress p185c-neu only (G8 and NV cells) and cells which overexpress EGFR only (NE19 cells), do not display any features of the transformed phenotype. The p185c-neu/EGFR double transfectants (GEr and two NVEr clones, M1 and K2) form foci in culture and result in tumor formation in nude mice (see Table 1).

Properties Of p185c-neu And EGFR In The Transformed Clones Overexpressing The Two Receptors

p185c-neu and the EGF receptors expressed on two NVEr clones, M1 and K2 were analyzed further to characterize the receptors. A 185 kd phosphoprotein was immunoprecipitated from M1, K2, Rat-1, and IEC18 (rat epithelial) cell lines with 7.16.4, a p185neu/p185c-neu specific monoclonal antibody. Similarly, a 170 kd phosphoprotein was immunoprecipitated from M1 and K2 clones with M294, an EGFR specific antibody. These data confirm that the transfected constructs properly encode the two receptors and that these proteins are phosphorylated.

EGFR-Mediated Phosphorylation of p185c-neu

EGF induces an EGFR-mediated phosphorylation of -185c-neu on tyrosine, as well as serine and threonine. p185c-neu isolated from cells treated with EGF has enhanced tyrosine kinase activity. To determine whether this interaction occurs in the double transfectants, M1 and K2 cells were metabolically labelled and incubated with an without 50 ng/ml EGF for 15 minutes at 37° C. The p185c-neu was immunopurified from cell lysates in the presence of Na_3VO_4 and electrophoresed using SDS-PAGE. EGF treatment increases the phosphorylation of p185c-neu.

Tyrosine Kinase Activity of Overexpressed p185c-neu

The tyrosine kinase activity of the overexpressed p185c-neu isolated from M1 cells treated with or without EGF was examined. p185c-neu was immunopurified from cell lines treated with or without EGF in the presence of Na_3VO_4 and used in an immune complex kinase assay. p185c-neu from EGF-treated cells exhibits 2–3 times higher autophosphorylation activity than p185c-neu isolated from untreated cells. These data confirm that this p185c-neu/EGFR interaction occurs in the doubly transfected cells.

Effects of Monoclonal Antibodies Directed Aqainst p185 neu and EGFR On The Tumorigenic Growth Of M1 and K2 Cells Implanted Into Nude Mice

M1 and K2 cells were NR6 cells transfected with cellular rat new and human EGF receptor cDNA. M1 cells express 1.3×10^5 molecules of p185c-neu and 1.4×10^5 molecules of EGF receptors, while K2 cells express 1.8×10^5 molecules of p185c-neu and 6.8×10^4 molecules of EGF receptors.

1×10^6 M1 or K2 cells were implanted subcutaneously in NCR nude mice, and the mice were subcutaneously treated with monoclonal antibodies to p185neu or the EGF receptor. The monoclonal antibodies used were Mab 7.16.4 (IgG2a), which is reactive with an extracellular domain of p185neu and capable of reverting the transformed phenotype of neu oncogene transformed cells in vitro and in vivo, and Mab 425, an IgG2a antibody which binds to the extracellular domain of human EGF receptor to inhibit EGF binding and induce EGF receptor down-regulation without stimulating EGF receptor tyrosine kinase activity.

1×10^6 cells were suspended in 0.1 ml of PBS or 0.1 ml of PBS containing 10 μg of monoclonal antibody and injected subcutaneously in the mid-dorsum of the mice. Each group of 6 mice received PBS or 10 μg of antibody administered subcutaneously on days 1 and 7. Growing tumors were measured with vernier calipers on every day after tumors appeared. Tumor volume was calculated as the product of tumor length, width and height. The student's T-test was used to determine the significance between groups. 30 μg of Mab 7.16.4 inhibited tumor growth of M1 and K2 cells by 80–90% ($p<0.05$). Treatment with 30 μg of MAb425 also inhibited the tumorigenic growth of M1 and K2 cells by 80–90%. Mab 7.16.4 and Mab 425 were found to have no effect on the growth of NIH3T3 cells transformed by Ha-ras. In addition, an irrelevant isotype matched monoclonal antibody, 9BG5 had no effect on the tumorigenic growth of M1 and K2 cells. In contrast to the effect of the two monoclonal antibodies, injection of high concentrations of EGF did not have any significant affect on tumor growth of either the M1 or K2 cells.

Effects of A combination of Monoclonal Antibodies Directed Against p185 neu and EGFR On The Tumorigenic Growth Of M1 and K2 Cells Implanted Into Nude Mice

M1 and K2 cells were NR6 cells transfected with cellular rat neu and human EGF receptor cDNA. M1 cells express 1.3×10^5 molecules of p185c-neu and 1.4×10^5 molecules of EGF receptors, while K2 cells express 1.8×10^5 molecules of p185c-neu and 6.8×10^4 molecules of EGF receptors.

1×10^6 M1 or K2 cells were implanted subcutaneously in NCR nude mice, and the mice were subcutaneously treated with monoclonal antibodies to p185neu or the EGF receptor. The monoclonal antibodies used were Mab 7.16.4 (IgG2a), which is reactive with an extracellular domain of p185neu and capable of reverting the transformed phenotype of new oncogene transformed cells in vitro and in vivo, Mab 425, an IgG2a antibody which binds to the extracellular domain of human EGF receptor to inhibit EGF binding and induce EGF receptor down-regulation without stimulating EGF receptor tyrosine kinase activity, and a combination of these two antibodies.

1×10^6 cells were suspended in 0.1 ml of PBS or 0.1 ml of PBS containing 10 μg of monoclonal antibody and injected subcutaneously in the mid-dorsum of the mice. Each group of 6 mice received PBS or 10 μg of antibody administered subcutaneously on days 1 and 7. Growing tumors were measured with vernier calipers on every day after tumors appeared. Tumor volume was calculated as the product of tumor length, width and height. The student's T-test was used to determine the significance between groups.

FIG. 1 shows the effects on tumor volume of the administration of antibody 7.16.4 (-◆-), antibody 425 (-■-) and a 10 μg mixture of 7.16.4 and 425 (-◈-). The PBS control is represented as (-⊡-). Mab 7.16.4 and 425 inhibited tumor growth by 70–85 percent as compared to the control. The mixture of Mabs 7.16.4 and 425, however, inhibited tumor growth by more than 90 percent as compared to the control.

Significance

Cotransfection of cells leading to overexpression of both p185c-neu and EGFR in the same cell mediates cell transformation in a specific manner. While overexpression of p185c-neu alone or EGFR alone is not sufficient to transform NIH3T3w and NR6 cell lines, the introduction and overexpression of EGFR in cells already overexpressing p185c-neu leads to transformation. The transfectants which overexpress both p185c-neu and EGFR from foci, produce colonies in soft agar, and form tumors in nude mice. The properties of the rat p185c-neu and the EGFR expressed on the doubly transfected cell lines were examined and these receptors were found to be indistinguishable from those seen in normal cells in terms of ligand binding affinities, molecular weights, and tyrosine kinase activities. Doubly transfected cells cause tumor formation when transplanted into mice. These tumors decrease in size when monoclonal antibodies to extracellular domains of p185neu and EGFR are injected into the tumors.

The data clearly show that two previously well characterized, normal cellular proteins (p185c-neu and EGFR) act in concert to transform NIH3T3 cells when coexpressed at high levels. The data also show that tumors formed from the doubly transfected cells synergistically regress when antibodies specific for extracellular domains of the two receptors interact with the cells.

I claim:

1. A method of treating mammalian tumors wherein the cells of such tumor express both 1) epidermal growth factor receptor and 2) p185neu, p185c-neu or a homologue of p185neu or p185c-neu, said method comprising

administering to a mammal having such a tumor at least one antibody specific for epidermal growth factor receptor and at least one antibody specific for p185neu, p185c-neu or a homologue of p185neu or p185c-neu or a homologue in an amount effective to reduce tumor growth, wherein said antibody specific for epidermal growth factor receptor is Mab425 and said antibody specific for p185neu, p185c-neu or a homologue of p185neu or p185c-neu is Mab 7.16.4.

2. The method of claim 1 wherein each of said antibodies is conjugated with a cytotoxic molecule.

3. The method of claim 1 wherein each of said antibodies is conjugated with a radioactive molecule.

4. A therapeutic composition for treatment of a mammalian cancer tumor having cells which express both epidermal growth factor receptor and p185neu, p185c-neu or a homologue of p185neu or p185c-neu comprising

at least one antibody specific for p185neu, p185c-neu or a homologue of p185neu or p185c-neu;

at least one antibody specific for epidermal growth factor receptor; and

a pharmaceutically acceptable carrier or diluent, wherein said antibody specific for epidermal growth factor receptor is Mab425 and said antibody specific for p185neu, p185c-neu or a homologue of p185neu or p185c-neu is Mab 7.16.4.

5. The composition of claim 4 wherein each of said antibodies is conjugated with a cytotoxic molecule.

6. The composition of claim 4 wherein each of said antibodies is conjugated with a radioactive molecule.

* * * * *

EXHIBIT E

EXHIBIT

US005824311A

United States Patent [19]

Greene et al.

[11] Patent Number: 5,824,311

[45] Date of Patent: Oct. 20, 1998

UPN-2105

[54] TREATMENT OF TUMORS WITH MONOCLONAL ANTIBODIES AGAINST ONCOGENE ANTIGENS

[75] Inventors: **Mark I. Greene**, Penn Valley, Pa.; **Jeffrey A. Drebin**, Baltimore, Md.

[73] Assignee: **Trustees of The University of Pennsylvania**, Philadelphia, Pa.

[21] Appl. No.: **347,019**

[22] Filed: **Nov. 30, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 573,527, Aug. 27, 1990, abandoned, which is a continuation-in-part of Ser. No. 126,572, Nov. 30, 1987, abandoned.

[51] **Int. Cl.**[6] **C07K 16/30**; C07K 16/28

[52] **U.S. Cl.** **424/138.1**; 424/143.1; 530/387.7; 530/388.22

[58] **Field of Search** 530/387.7, 388.22; 424/138.1, 143.1; 435/240.27, 172.2, 70.214

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Primary Examiner—Toni R. Scheiner

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[57] **ABSTRACT**

A method of treating certain mammalian tumors with monoclonal antibodies is provided. Monoclonal antibodies specific to distinct epitopes of p185, the translation production of the neu oncongene, are provided, and these are then contacted with the tumor antigen under conditions which allow binding of the antibodies to a degree sufficient to inhibit tumor growth. The monoclonal antibodies act synergistically thus enhancing their anti-tumorigenic effect upon the tumor. An injectable composition for treating certain mammalian tumors with monoclonal antibodies and methods for diagnosing mammalian cancer tumors which express the protein p185 on the surface of the cells are also disclosed.

24 Claims, 2 Drawing Sheets

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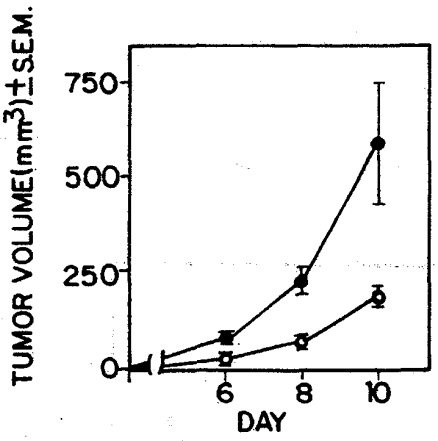

FIG. 1A

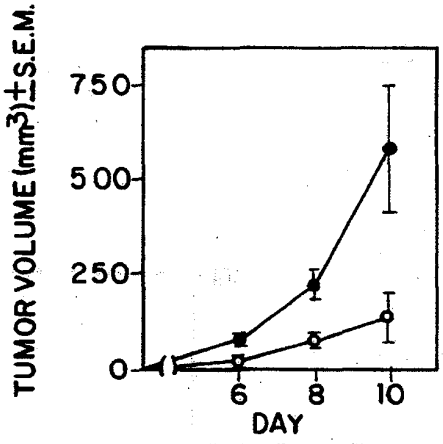

FIG. 1B

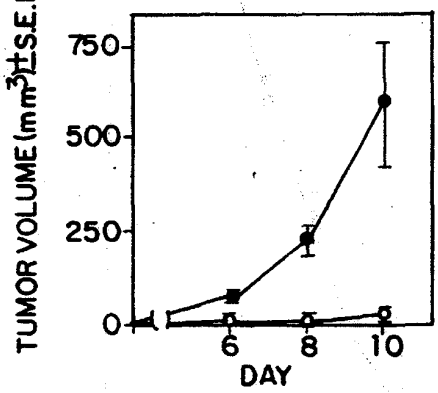

FIG. 1C

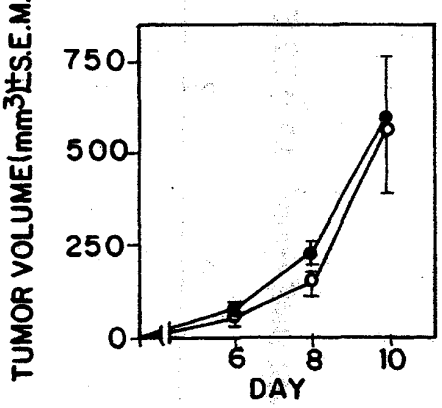

FIG. 1D

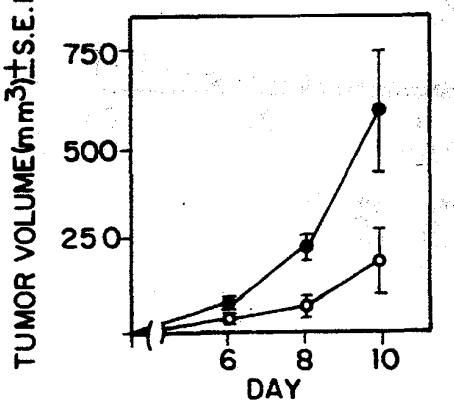

FIG. 1E

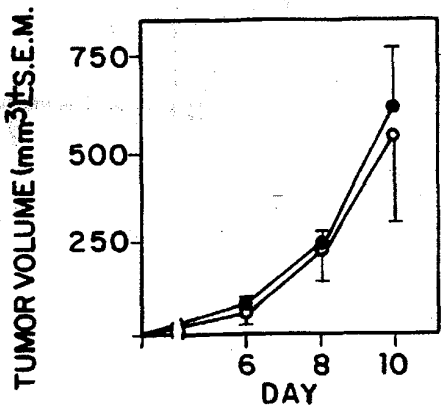

FIG. 1F

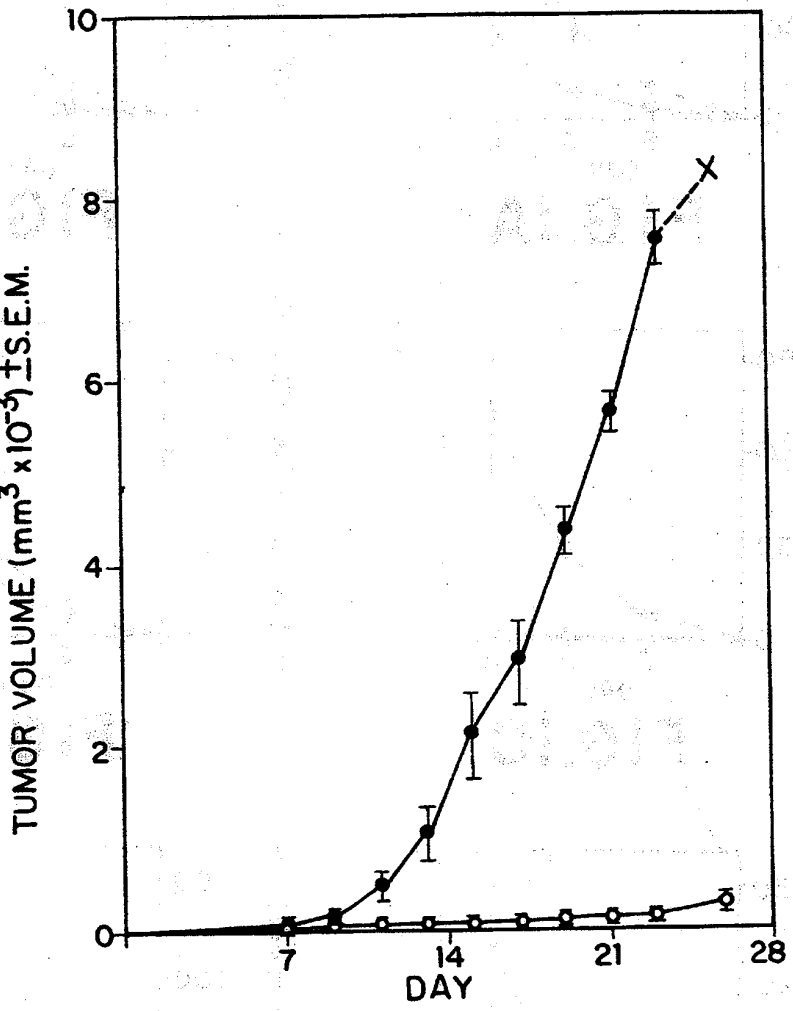

FIG. 2

TREATMENT OF TUMORS WITH MONOCLONAL ANTIBODIES AGAINST ONCOGENE ANTIGENS

REFERENCE TO RELATED APPLICATIONS

This is a continuation of application Ser. No. 07/573,527, filed Aug. 27, 1990, now abandoned, which is a continuation-in-part of application Ser. No. 07/126,572, filed Nov. 30, 1987, now abandoned.

FIELD OF THE INVENTION

This invention is directed to treatments and diagnoses for mammalian tumors. More particularly this invention is directed to methods of treating and diagnosing mammalian cancer tumors that employ antibodies.

BACKGROUND OF THE INVENTION

Huge amounts of time and money have been spent searching for mammalian cancer tumor treatments. Current tumor treatments rely on the cytotoxic effects of drugs and radiological therapy. Although these treatments bring remission and cure to some patients, they unfortunately have serious side effects because they kill not only tumor cells but also normal non-tumorous cells. There exists a great need for mammalian tumor treatments which affect only the tumor cells.

Tumor treatments employing immunotherapy by passive transfer of monoclonal antibodies, lymphokines, and/or cellular effectors into the tumor-bearing host has shown promise in laboratory and clinical trials. Most studies of monoclonal antibody-mediated immunotherapy have utilized monoclonal antibodies generated against random structures on the malignant cell surface, and have depended upon the ability of immunologic effector mechanisms in the tumor bearing host to eradicate antibody-coated tumor cells.

U.S. Pat. No. 4,522,918 to Schlom et al. discloses a cancer treatment using monoclonal antibodies directed at surface antigens of human mammary adenocarcinoma cells. These antibodies are activated only by tumor cells from human mammary cells and not by apparently normal human tissues. They were prepared from mouse spleen cells which had been immunized with human metastatic mammary carcinoma cells. The mouse spleens were fused with NS-1 myeloma cells to generate hybridoma culture which secreted antibodies reactive with surface antigens of human mammary adenocarcinoma cells but not with surface antigens of normal cells.

U.S. Pat. No. 4,444,744 to Goldenberg discloses radiolabeled monoclonal antibodies to tumor cell surface antigens to locate, diagnose and stage tumors having such antigens of their cell surfaces. Radiolabeled monoclonal antibodies to a tumor-associated or tumor-specific antigen is injected parenterally into a human subject along with an indifferent immunoglobulin from the same or different species as that used to prepare the specific antibody. The specific and indifferent antibodies are labeled with different isotopes. The level of activity of the labeled indifferent immunoglobulin being used to determine the distribution of background activity due to non-targeted specific antibody, the background activity being subtracted from the total activity of specific antibody, whereby the activity of substantially only the targeted tumor-localized specific antibody is determined and the tumor is thereby detected and localize U.S. Patent to Gansow et al. discloses the use of metal chelate conjugated monoclonal antibodies to diagnose and treat cancer cells.

Here monoclonal antibodies specific for a cell or antigen on the cell surface are chelated with a metal which may be radioactive, exhibit fluorogenic properties, exhibit paramagnetic properties or other property. The antibody-metal chelates are injected into to body where they attach to the target cells and kill them or tag the cells so they can be picked up in diagnostic tests.

Capone et al., JNCI 72: 673–677, (1984), investigated the relationship between antigen density and immunotherapeutic response elicited by monoclonal antibodies against solid tumors. These investigators used monoclonal antibodies specific against human breast cancer. It was found that passively administered monoclonal antibody can be effective in producing a tumor regression response against solid tumors. Tumoricidal response with monoclonal antibody appeared to be exponentially related to the density of the antigen on the cells.

In an effort to achieve more potent anti-tumor effects, scientists have begun producing antibodies which are specific for structures necessary for neoplastic cell growth and/or which are capable of directly interfering with neoplastic cell functions. U.S. Pat. No. 4,443,427 to Reinnerz et al. discloses a monoclonal antibody specific to a mature human T cell surface antigen. These monoclonal antibodies are capable of selectively binding mature human T cells and rendering them inactive in vivo and failing to induce the proliferation or activation of human lymphocytes.

Bast et al., Cancer Research 45: 499–503, (1985), studied the in vitro elimination of malignant clonogenic cells from human bone marrow using multiple monoclonal antibodies and complement. These researchers used monoclonal antibodies which react with acute lymphoblastic leukemia cells and in addition to testing each monoclonal antibody singly for antitumorigenic effect, they tested combinations of these monoclonal antibodies. Several combinations of antibodies were found to be more effective than single antibodies; however, a combination of three antibodies was not significantly more effective than the optimal combinations of two antibodies for eliminating tumor cells.

Herlyn et al., Journal of Immunology 134: 1300–1304, (1985), investigated the effects of mixtures of monoclonal antibodies on tumor growth in vitro and in vivo. Some monoclonal antibodies they tested exerted no tumoricidal effect in vitro when used singly, however, two mixtures (two antibodies in each mixture) which were not tumoricidal separately were effective in lysing melanoma cells in vitro when used together. These mixtures of monoclonal antibodies did not, however, affect the growth of these tumors in vivo. Two of the antibodies used by Herlyn et al. bound to different epitopes of the p97 antigen on the melanoma cells. However, when tested as a mixture, they did not act synergistically to reduce tumor growth. They attributed this lack of synergistic effect to stearic hindrance of binding of effector cells to antibody molecules bound to the same antigen. In another study, Ralph and Nakoinz, J. Leuk. Biol. 35: 131, (1984), reported that mixtures of monoclonal antibodies binding to different molecules on the tumor cells surface cooperated in the tumor cell lysis, whereas monoclonal antibodies binding to the same molecule did not.

Thus there is a long-felt need for improved treatments for cancer which affect only the tumor cells. Known treatments have proved to be ineffective in treating cancer tumors in vivo.

Malignant cells display a variety of in vitro characteristics that distinguish them from normal cells. These characteristics, collectively known as the transformed

phenotype, include anchorage-independent growth, decreased serum requirements, rounded cellular morphology, increased hexose uptake, loss of microfilaments, increased plasminogen activator secretion, decreased cell surface fibronectin, and increased sensitivity to the drug ouabain. Anchorage-independent growth, as determined by the formation of colonies in soft agar, is the most reliable parameter of the transformed phenotype because it is the phenotypic property most tightly linked with tumorigenic behavior in vivo.

Recent studies in the molecular genetics of cancer indicate that certain genes known as oncogenes may play a role in the transformation of some cells from their normal condition to a cancerous condition. Proto-oncogenes, genes closely related to these genes, are found in somatic cells of all eukaryotic species examined and have been highly conserved in evolution; it is thought that proto-oncogenes normally play critical roles in cellular growth and development. Oncogene amplification and chromosomal rearrangements involving oncogenes have been detected in a large number of tumors. Furthermore some tumors have been shown to contain activated oncogenes which, in DNA transfection assays, are capable of conferring neoplastic properties upon non-neoplastic rodent fibroblast cell lines. Collectively these studies suggest that alterations in proto-oncogene structure and function play a critical role in the development of neoplasia.

Although most oncogene-encoded proteins reside in the nucleus or the cytoplasm, some oncogenes encode proteins that express antigenic sites on the cell surface. For example, the erbB, fms and ros oncogene products are transmembrane glycoproteins that possess extracellular domains. The sis oncogene product may also exist in a membrane associated form on the surface of transformed cells. Another oncogene which encodes a protein that exposes antigenic sites on the surface of transformed cells has been identified by transfection of DNA from ethyl nitrosourea-induced rat neuroblastomas into NIH3T3 cells. This oncogene has been termed neu. The neu gene has been found to be amplified in some human tumors, particularly those of the breast, suggesting that this gene may play a role in the etiology of human cancer.

The neu oncogene encodes a cell surface protein on rat cells transformed by it. The protein encoded by the neu oncogene is a 185 kDa transmembrane glycoprotein with tyrosine kinase activity, generally known by the name p185. The neu gene is closely related to the epidermal growth factor (EGF) receptor gene in structure. It is thought that p185 is a receptor for an, as yet, unidentified growth factor.

The neu oncogene and p185 have also been found active in human adenocarcinomas including breast, lung, salivary gland and kidney adenocarcinomas, as well as prostate neuroblastoma.

In human primary breast cancers amplification of the neu oncogene was found in about 30% of all malignant tumors examined. Increased stage of malignancy, characterized by large tumor size and increased number of positive lymph nodes as well as reduced survival time and decreased time to relapse, was directly correlated with an increased level of amplification of the neu gene. The neu protooncogene is expressed at low levels in normal human tissues.

SUMMARY OF THE INVENTION

The present invention provides methods for the treatment of mammalian cancer tumors having cells which express a translation product of the neu oncogene on their surfaces. In accordance with the invention, a first antibody specific for a first epitope of the translation product and a second antibody specific for a second epitope of the translation product, the combination of first and second antibodies being selected to produce synergistic inhibition of tumor growth, are contacted with the cells under conditions which allow the antibodies to bind to the translation product on the surfaces of the cells to a degree sufficient to inhibit growth of the tumor. In accordance with preferred embodiments of the invention, the translation product of the neu oncogene is p185, a transmembrane glycoprotein having tyrosine kinase activity and a molecular weight of about 185,000 daltons as determined by carrying out electrophoresis on the glycoprotein and comparing its movement with marker proteins of known molecular weight. The exact mechanism of the antitumorigenic effect is not known. Experiments have shown that antibody binding to p185 leads to a reversion of the cancerous phenotype to the non-cancerous phenotype. Anti-p185 antibodies selectively inhibit the neo-plastic behavior of neu transformed cells, without in any way affecting cell viability.

Treatment of tumors with antibodies has been done by others; however, these treatments used monoclonal antibodies singly or in combination with a cytotoxic agent. The present invention shows that antibodies specific for a cell surface structure necessary for malignant cell growth can be curative in vivo. In no studies done previously were antibodies used that interacted with elements necessary for growth. The present invention employs two or more monoclonal antibodies to distinct domains of the same tumor antigen. These antibodies bind to the protein and the effect produced by the antibodies is synergistic. The proper combination of antibodies produces antitumorigenic effects beyond that expected by the mere binding of two antibodies to the tumor antigen. The extent of the tumorigenic effect produced by the proper combination was quite unexpected and could not be anticipated. A few researchers have used mixtures of antibodies to tumor antigens to attempt to reduce tumor size but they were not successful in producing these effects in vivo and the mixtures of antibodies did not act synergistically. Ralph and Nakoinz have succeeded in showing cooperation between monoclonal antibodies binding to different molecules of the tumor cell surface to lyse tumor cells in vitro. These researchers could not show cooperation between antibodies when they used mixtures of antibodies specific for the same molecule. This may relate to the fact that the tumor antigens in question are not intimately involved in malignant cell growth. The antigens studied to date may be expressed as a consequence of malignancy, but do not determine malignant growth. The neu oncogene product specifically and uniquely determines malignant growth on its own.

Accordingly, the invention provides a finely tuned treatment for those mammalian tumors which express the tumor antigen, p185, on their cell surfaces. This treatment is an improvement over tumor treatments already in use because the antibodies affect only tumor cells, unlike mammalian cancer tumor treatments currently in use which affect all cells. The treatment of the invention can thus be expected to reduce or eliminate the serious side effects of mammalian cancer tumor treatments because it does not interfere with any part of the body except the tumor. The invention also provides monoclonal antibodies for the treatment of mammalian cancer tumors that express products of the neu oncogene on their surfaces.

The invention further provides an injectable composition for treatment of a mammalian cancer tumor having cells

which express a translation product of the neu oncogene on the surfaces of the cells. In accordance with the invention, the composition comprises a first antibody specific to a first epitope of a translation product, a second antibody specific to a second epitope of a translation product, the combination of first and second antibodies being selected to produce synergistic inhibition of tumor growth, and a pharmaceutically acceptable injection vehicle.

The invention additionally provides methods for diagnosing mammalian cancer tumors which express the protein p185, a translation product of the neu oncogene on the tumor cell surfaces. In accordance with one embodiment of the invention, tumors can be diagnosed by contacting tissue portions of the tumor with an antibody specific for a translation product of the neu oncogene, the antibody being labeled with an indicator. The antibody binds to a translation product of the neu oncogene present in the cells of the tissue portion. The indicator is then detected. In preferred embodiments of the invention, the indicator comprises biotinylated horse anti-mouse immunoglobulin and streptavidin-biotinylated-peroxidase. The indicator is detected by contacting the indicator with a chromogenic substrate which preferably comprises 3,3'-diaminobenzidine, hydrogen peroxide and imidazole. The chromogenic substrate is then detected by microscopy.

In accordance with another embodiment of the invention, a mammalian cancer tumor having cells which express a translation product of the neu oncogene on the surfaces of the cells can be diagnosed by contacting a radiolabeled nucleic acid probe with nucleic acid prepared from the tumor causing the probe to bind to nucleic acid coding for the neu oncogene translation product and detecting the radiolabeled probe. In preferred embodiments of the invention, the radiolabeled probe comprises a nucleic acid fragment of neu oncogene and a radiolabel. The radiolabeled probe is contacted with RNA prepared from the tumor cells and the radiolabeled probe is then detected autoradiographically.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the inhibition of tumorigenic growth of neu-transformed cells after intravenous administration of anti-p185 monoclonal antibodies.

FIG. 2 shows the effects of anti-p185 antibody treatment of tumor-bearing mice.

DETAILED DESCRIPTION OF THE INVENTION

The injectable composition for treatment of mammalian cancer tumors which express p185 on the surface of the cells comprises a mixture of antibodies specific for different domains or sites on the p185 molecule and a pharmaceutically acceptable injection vehicle. The antibodies are chosen from antibodies made according to the procedures described in detail below or other conventional methods for producing monoclonal antibodies. The injection vehicle can be an injection vehicle known in the art such as sterile saline.

Mammalian cancer tumors which express p185 on the surface of the cells can be diagnosed using immunohistochemical and nucleic acid probe/autoradiographical procedures. A detailed description of the preferred embodiments of these procedures is set forth below.

A detailed description of the preferred embodiments of the invention is set forth below.

Experimental

Antibodies specific for different sites of p185.

Transformed Cell Lines

B104-1-1 is a neu oncogene transformed NIH3T3 cell line derived by passing rat neuroblastoma transforming DNA sequences through two cycles of transfection in NIH3T3 cells. XHT-1-1a is a Ha-ras-transformed NIH3T3 cell line. Cells are cultured in 100 mm tissue culture dishes (Costar), in 10 ml of Dulbecco's Modified Eagle's Medium (DMEM, K.C. Biologicals) supplemented with 10% fetal calf serum, 1% pen-strep-fungizone mixture (M.A. Bioproducts) and 100 ug/ml gentamicin sulfate (M.A. Bioproducts). Cell cultures are maintained in a humidified 5% CO2 incubator at 37 C. and replaced from stock every 2–3 months.

Experimental Animals

C3H and [C3H×DBA/2] F1 (C3D2 F1) mice were obtained from the Jackson Laboratory, Bar Harbor, Me. Inbred congenitally athymic Balb/c nude (nu/nu) mice were obtained from the National Cancer Institute animal colony (San Diego, Calif.). Animals used in the experiments are maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals of the Institute of Animal Resources, National Research Council (DHEW publication number (NIH) 78-23, revised 1978).

Isolation of Hybridomas Secreting Monoclonal Antibodies Reactive with Neu-Transformed Cells

C3H/HeJ mice are repeatedly immunized with NIH 3T3 transfectants transformed by the neu oncogene (cell line B104-1-1), emulsified in Freund's adjuvant. Spleens from immune mice are fused with the aminopterin-sensitive NS-1 myeloma line, and hybridomas are selected in hypoxanthine-aminopterin-thymidine media. Culture supernatants from growing hybridomas are initially screened for the presence of antibody capable of binding B104-1-1 cells by indirect immunofluorescence using fluorescence activated cell sorting (FACS). Positive supernatants are then tested for specificity by determining whether they contain antibody capable of binding normal NIH 3T3 cells, or NIH 3T3 cells transformed by transfection with Harvey sarcoma virus proviral DNA (cell line XHT-1-1a).

Isotype Analysis of Monoclonal Antibodies

The heavy chain isotypes of the monoclonal antibodies characterized here are determined by double immunodiffusion in agar according to the method of Ouchterlony, in Hudson, L and F. C. Hay, eds., *Practical Immunology*, Blackwell Scientific Publications, London, p. 117, which is specifically incorporated herein.

Purification of Monoclonal Antibodies

Hybridoma cells are washed several times in HBSS and injected into pristine primed, 400 rad irradiated, C3D2F1 mice to induce ascites fluid production. When the mice develop significant ascites, the fluid is removed by aspiration with a 19 gauge needle and hybridoma cells and debris are removed by centrifugation at 1000×g. The clarified ascites fluid is then stored at −70 C. prior to purification, or is purified immediately. Purification is performed according to the method of Drebin et al. in *Immunology and Cancer* (M. L. Kripke and P. Frost, eds.) University of Texas Press, Austin, Tex., p. 277 which is specifically incorporated herein.

II. Specificity of Antibodies Flow Cytometry

Cells are removed from dishes with buffered EDTA (Versene; Gibco) and washed twice in FACS medium

(Hank's balanced salt solution (HBBS; Gibco) supplemented with 2% fetal calf serum (FCS), 0.1% sodium azide and 10 mM HEPES); 1×10^6 cells in 0.1 ml FACS medium are incubated with 0.1 ml of hybridoma culture supernatant for 1 hr at 4 C. Cells are washed twice with FACS medium, and incubated with 0.1 ml fluorescein isothiocyanate (FITC) -conjugated rabbit-anti-mouse immunoglobulin (Miles) diluted 1:50 in FACS medium for 1 hr at 4 C. Cells are then washed twice in FACS medium and fixed in 2% paraformaldehyde-phosphate-buffered saline (PBS). Samples are run on an Ortho 2150 Cytofluorograph using the logarithmic amplifier. Each sample contains 10,000 cells per sample.

Cyanogen Bromide Coupling of Antibodies to Sepharose Beads

CNBr-activated Sepharose 4B beads are swollen in 1 mM HCl, and then mixed with purified antibodies in coupling buffer (0.5M NaCl, 0.1M $NaHCO_3$, pH 8.3) at a ratio of 2 mg immunoglobulin (1 mg per ml) per ml of activated beads. The mixture is rotated overnight on an end-over-end mixture at 4 C., and then unreacted sites are blocked with 0.2M glycine pH 8.0 for 2 hours at room temperature. The beads are then poured onto a sintered glass filter and washed with three cycles of 100 bead volumes of coupling buffer, 10 bead volumes of 3.5M $MgCl_2$, 100 bead volumes of coupling buffer to wash away excess adsorbed proteins. Non-specific protein binding to the antibody coupled beads is blocked by a brief wash in sterile DMEM containing 10% fetal calf serum. The beads are then washed in PBS and stored in PBS containing 0.1% sodium azide at 4 C. until they are used in immunoprecipitation experiments.

All of the monoclonal antibodies which specifically bind to the surface of neu-transformed cells are reactive with the p-185 molecule encoded by the neu oncogene. These monoclonal antibodies specifically precipitate p185 from metabolically labeled lysates of neu-transformed cells.

Immunoprecipitation of p185 from Metabolically Labeled B104-1-1 Cell Lysates

For labeling with ^{35}S-cysteine 10^6 cells are seeded in 100 mm culture dishes and labelled for 18 hr in 2 ml minimal essential medium (MEM) containing 0.1 the usual amount of cysteine, 2% dialyzed fetal calf serum and 500 uCi ^{35}S-cysteine (77 Ci mmol^{-1}; NEN). For labeling with ^{32}P, 3×10^5 cells are seeded in 60-mm tissues culture dishes and incubated for 18 hr in 0.8 ml phosphate-free Dulbecco-Vogt modified Eagle's medium containing 4% fetal calf serum and 0.4 mCi ^{32}P (carrier-free; NEN). Cells are lysed in phosphate-buffered RIPA buffer containing 1 mM ATP, 2 mM EDTA and 20 mM sodium fluoride, and immunoprecipitates are prepared and washed according to Sefton et al. Virology 28: 957–971 (1979), which is specifically incorporated herein. One third of each lysate is incubated with 1 ul of normal mouse serum or 60× concentrated 7.16.4 culture supernatant at 4 C. for 60 min. Sheep anti-mouse immunoglobulin (1 ul; Cappel) is added to each sample and incubation continued for 30 min. Immune complexes are pelleted using fixed Protein A-bearing *Staphylococcus aureus* and washed. Samples are analyzed by SDS-polyacrylamide gel electrophoresis in 7.5% acrylamide—0.17% bis-acrylamide gels. The gels are treated for fluorography and exposed to preflashed Kodak X-Omat AR film for 10 days.

Anchorage-Independent Growth Assays and Results

Anchorage independent growth as assessed by determining the colony-forming efficiency of cells suspended in soft agar. Assays are conducted using 60 mm tissue culture dishes containing a 6 ml free feeder layer and a 1 ml top layer in which the cells are suspended. Feeder layers consist of 0.24% agarose RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. When antibody is added to soft agar cultures, it is incorporated into the top layer only. Cultures are fed after 7 days with 1 ml of DMEM containing 10% fetal calf serum and antibiotics, and the same amount of antibody that was added on day 1. On day 13, the cultures are fed 1 ml of HBSS containing 1 mg/ml p-iodonitrotetrasolium violet (INT, Sigma) to stain colonies. The next day colonies >0.5 mm are counted using a dissecting microscope and a calibrated template. Each group represents the mean of triplicate samples.

One of the most stringent characteristics distinguishing malignant from non-malignant cells is the capacity for anchorage-independent growth. Exposure of neu-transformed cells to the p185 specific monoclonal antibody 7.16.4 causes the down-modulation of p185 from the cell surface and results in loss of the capacity for anchorage-independent growth. The ability of each of the p185 specific antibodies to inhibit the anchorage-independent growth of neu-transformed cells is shown in Table 1. As shown in Table 1 below, all of the anti-p185 monoclonal antibodies are able to cause over fifty percent inhibition of the anchorage-independent growth of B104-1-1 cells at doses of less that 1 ug per dish. The potency of the different anti-p185 antibodies in inhibiting anchorage-independent growth parallels their relative affinity for binding B104-1-1 cells, with antibody 7.16.4 having the highest affinity and antibody 7.21.2 having the lowest affinity. The relative affinity is deduced by the saturable binding curves of the various purified antibodies for p185 expressed on B104-1-1 cells. In addition, the antibodies identify three distinct domains of p185. Thus, 7.16.4, 7.9.5, and 7.21.2 react with independent epitopes of the extra cytoplasmic portions of p185. Hence, the effects observed can not be attributed to the binding of the different monoclonals to the same site.

TABLE I

Anti-p185 Monoclonal Antibodies Inhibit The Anchorage-Independent Growth Of neu-transformed Cells

		Anchorage-Independent colonies[a] (percent inhibition)			
Antibody	(specificity)	0	100 ng	1 ug	10 ug	100 ug
None		25 ± 2.2				
7.5.5	(anti-p185)		15 ± 0.3 (40)	10 ± 2.5 (60)	6 ± 1.0 (76)	4 ± 0.9 (84)
7.9.5	(anti-p185)		14 ± 1.2	9 ± 0.9	7 ± 0.6	0.3 ± 0.3

TABLE I-continued

Anti-p185 Monoclonal Antibodies Inhibit
The Anchorage-Independent Growth Of neu-transformed Cells

		Anchorage-Independent colonies[a] (percent inhibition)			
Antibody	(specificity)	0 100 ng	1 ug	10 ug	100 ug
		(44)	(64)	(72)	(99)
7.16.4	(anti-p185)	3 ± 3.3	1 ± 0.6	1 ± 0.6	0.7 ± 0.3
		(88)	(96)	(96)	(97)
7.16.4	(anti-p185)	16 ± 1.9	3 ± 0.9	1 ± 0.3	0.3 ± 0.3
		(36)	(88)	(96)	(99)
7.21.2	(anti-p185)	25 ± 2.0	11 ± 0.9	12 ± 1.9	12 ± 1.2
		(0)	(56)	(52)	(52)
9BG5	(IgG2a, anti-reovirus)	21 ± 1.5	23 ± 2.0	21 ± 3.2	22 ± 1.3
		(16)	(8)	(16)	(12)
87.92.6	(IgM, anti-beta andrenergic receptor)	22 ± 1.2	26 ± 2.6	29 ± 2.9	23 ± 1.2
		(12)	(<0) (<0)	(8)	

[a]colonies > 0.5 mm were counted using a dissecting microscope after 14 days, described in Materials and Methods.

In contrast to the effects of anti-p185 antibodies, two control monoclonal antibodies fail to significantly inhibit the anchorage-independent growth of neu-transformed cells even at 100 ug per dish (Table I). It is important to note that one of these control antibodies, 87.92.6, is reactive with a beta-adrenergic like receptor on B104-1-1 cells and shows significant binding to these cells by immunofluorescence, but has no effect on their anchorage-independent growth. This demonstrates that the effects of the anti-p185 monoclonal antibodies on the anchorage-independent growth of neu-transformed cells does not simply result from antibody binding the cell surface, but reflects a specific cytostatic effect resulting from antibody binding to specific domains of the p185 molecule.

The ability of anti-p185 monoclonal antibodies to inhibit the growth of neu-transformed cells occurs exclusively under conditions that are selective for neoplastic behavior, such as when the cells are suspended in soft agar.

Effect of Anti-p185 Monoclonal Antibodies on Adherent Growth in Ten Percent Fetal Calf Serum

Adherent growth in ten percent fetal calf serum, which is a property shared by non-neoplastic cells as well as neoplastic cells, is unaffected by anti-p185 antibodies. Antibody 7.16.4 has no effect on the adherent growth of neu-transformed cells in liquid medium, even at concentrations that inhibit the anchorage-independent growth of neu-transformed cells by >95%. Because the expression of p185 is not significantly affected when cells are grown under varying conditions, differential expression patterns of p185 is not considered a significant element in the failure to observe affects of anti-p185 antibodies on cells grown in liquid 27 media. Collectively, these studies demonstrate that anti-p185 antibodies selectively inhibit the neoplastic behavior of neu-transformed cells, without in any way affecting cell viability.

Effect of Anti-p185 Antibody on Rats

Intravenous injection of up to 4mg of purified antibody 7.16.4 per rat has no discernable toxic effects on BDIX rats or on the offspring of BDIX rats when given on day 16 of pregnancy in toxicity studies.

Antibody Dependent Complement-Mediated Cytotoxicity Assays

Tumor targets are removed from culture dishes and incubated in a volume of 0.5 ml with 200 uCi of $Na^{51}CrO_4$ (New England Nuclear) at 37 C. for 60 minutes. The cells are then washed three times in Hank's Balanced Salt Solution (HBSS), counted in a hemocytometer, and diluted to 10^5 cells per ml. 100 ul of cell suspension (10^4) cells) is added to wells of a 96 well microtiter plate. 50 ul of appropriately diluted rabbit complement is added to each well. Next, 50 ul aliquots containing the appropriate amounts of purified monoclonal antibodies are added to each well. Appropriate control wells containing cells alone, cells plus antibody only, cells plus complement only and cells plus Triton x-100 detergent (to effect maximal cell lysis) are set up in parallel. Microtiter plates are incubated at 37 C. for 1 hr and then centrifuged at 1000×g for 10 minutes. 100 ul of supernatant is collected from each well, and ^{51}Cr released into the supernatant is assayed in a gamma counter. Specific release is calculated from the formula: x-C/M-C, where x represents the ^{51}Cr activity in counts per minute of the experimental wells, C represents the ^{51}Cr activity of the complement control wells, and M represents the maximal ^{51}Cr activity released from detergent containing wells. Release from wells containing antibody without complement is never significantly greater that the release from wells containing medium only. All experimental groups represent the mean of triplicate samples. Individual samples never varied from the mean by more than 10%.

All of the anti-p185 antibodies (7.5.5, 7.9.5, 7.17.4, 7.16.5, and 7.21.2) exert a cytostatic effect on the growth of neu-transformed cells suspended in soft agar. In order to identify additional mechanisms by which anti-p185 monoclonal antibodies might exert anti-tumor effects, we examined their abilities to kill tumor cells in vitro in the presence of rabbit complement. Purified immunoglobulin from the 6.16.4 hybridoma is able to lyse neu-transformed cells in the presence of complement in a one hour ^{51}Cr release assay at immunoglobulin concentrations as low as 5 ng/ml. The ability of antibody 7.16.4 to kill neu-transformed cells is completely dependent on the addition of complement since purified immunoglobulin does not exert cytotoxic effects in the absence of complement in either short term in vitro ^{51}Cr release assays or longer term cell cultures. The anti-p185 antibodies, 7.5.5 and 7.16.5 are also able to cause significant lysis of the neu-transformed NIH3T3 cell line B104-1-1 in the presence of complement. There is no killing of the control cell line XHT-1-1a by any of the monoclonal anti-p185 antibodies.

Antibody Dependent Cell-Mediated Cytotoxicity Assays

Tumor targets are labeled with $Na^{51}CrO_4$ as described above. 1×10^4 labeled tumor cells, 20 ug of antibody 7.16.4,